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CANCER OF THE LUNG, TRACHEA AND LARYNX IN SINGAPORE

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CANCER of the lung is held to be increasing in incidence, probably on a world-wide basis. This paper presents data on cancers of the lung, trachea and larynx based on the post-mortem protocols of the University and Government Departments of Pathology, Singapore, and on the reports of the Registrar-General, Singapore, in the hope that the information adduced will prove useful as a base-line for subsequent epidemiological cancer research in this area.

METHODS AND MATERIALS

The post-mortem records of all cases of cancer of the lung, trachea and larynx for the years 1948–58 inclusive were individually scrutinised and the relevant data entered on a pro-forma for subsequent analysis. Nearly all post-mortems in Singapore are performed by the staffs of the two departments of Pathology. These embrace both hospital deaths and deaths coming within the aegis of H.M. Coroner, Singapore, i.e. all violent, sudden and unnatural demise.

The age and sex distribution of both the Singapore and the post-portem population for the period under review have been published previously, and the probable bias and error inherent in the material indicated (Muir, 1959). Briefly, although there is a multiracial population of Malays, Chinese, Indians and Others the post-mortem rates for these races are not proportional to their absolute numbers, due to religious and other factors, and further, there is some aversion, particularly among the Malays, who tend to live in rural Singapore, to seek medical advice. It is probably true to say that this latter factor will decrease in importance over the next decade.

The lung

There were 173 cases of primary cancer of the lung. This represents 0·75 per cent of the 22,997 post-mortems carried out in 1948–58, or if the 11,525 children under the age of 11 years be excluded, 1·5 per cent.

There were 139 males. The mean age at death was 53·4 $\pm$ 10·7 years. (One boy aged 11 years, and 2 males of unknown age are not included in this figure.) Of these 129 were Chinese, 5 were Europeans (mean age 55 years), 3 were Tamil Indians (mean age 43 years) and 2 were Malay (mean age 37 years).

Of the 34 females, whose average age at death was 53·8 $\pm$ 13·7 years, 31 were Chinese. There were also one Eurasian, one Japanese and one Tamil Indian.

The male/female ratio is thus 4 to 1. The age distribution by sex for the Chinese is shown in Fig. 1, and is compared with that of a series of 1749 lung cancers seen at the Brompton Hospital, London (Bignall, 1958).

It will be seen that although the maximum incidence in London and Singapore is in the decade 50–59, there are relatively more deaths before the age of 40 and between 40–49 in Singapore.

The primary tumour was in the left lung in 64 (37 per cent), in the right in 104 (60 per cent), and at the tracheal bifurcation in 2 (1 per cent). In a further

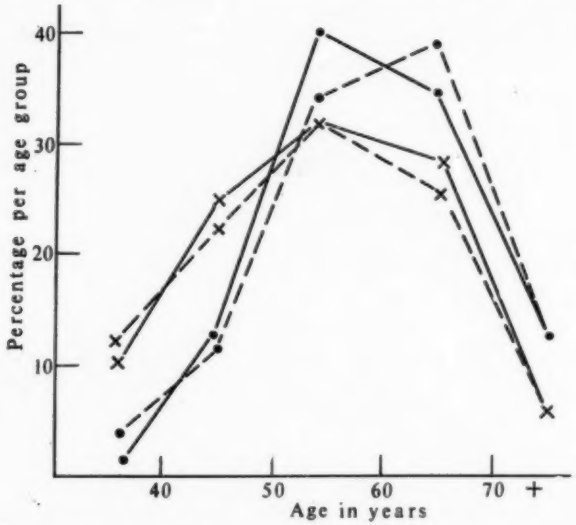

FIG. 1.—Comparison of the age distribution, by sex, of the Singapore Chinese with lung cancer, with a series from the Brompton Hospital, London (Bignall, 1958).

×———× Chinese males. ●—+—● English males.
×- - - - - - - × Chinese females. ●- - - - - - - ● English females.

2 (1 per cent) it was impossible to state in which lung the primary tumour arose. There was one case of pleuro-endothelioma, in the boy aged 11 years mentioned above.

In the left lung 26 per cent of tumours arose from the main bronchus, 48 per cent were found in the upper lobe, 20 per cent in the lower lobe. In the remaining 6 per cent the site was not, or could not, be specified. In the right lung 30 per cent arose from the main bronchus, 30 per cent were found in the right upper lobe, 5 per cent in the middle lobe and 28 per cent in the lower lobe. In 7 per cent the site was not specified. These figures are in general accord with the observations of Bryson and Spencer (1951).

Intrapulmonary metastases were very common, and in 20 per cent involved the opposite lung. Proportionately, neither lung seemed to have spread to its fellow more often than the other. Ochsner and de Bakey (1942) recorded metastasis in the opposite lung in 23 per cent.

The ratio between tumours of the right and left lungs is as in most occidental reports (Ochsner and de Bakey, 1942).

Metastases were common. The mediastinal glands were grossly involved in 66 per cent, the cervical and supraclavicular glands in 16 per cent. Spread to the upper abdominal nodes was recorded in 12 per cent. These figures are somewhat lower than those of Ochsner, Dixon and de Bakey (1945).

Pericardial metastases were present in 20 per cent; haemopericardium, often exceeding 200 ml., in a further 6 per cent; cardiac secondaries were seen in 2 per cent. The superior vena cava was invaded in 10 per cent and significantly compressed in a further 10 per cent. The pulmonary artery, the aorta, and other great vessels were invaded in 3 per cent. One case of direct fistula between aorta and bronchus was recorded. Bony spread (15 per cent) was most often seen in the ribs, the femur and the frontal bones of the skull. Femoral fracture occurred in 6 per cent.

Pancreatic, thyroid, splenic and peritoneal spread occurred in 3 per cent.

Onuigbo (1957) studying 1000 lung cancer autopsies found suprarenal metastases in 38·5 per cent, hepatic in 42·6 per cent and kidney in 17·3 per cent, the adrenal and liver figures being 10 per cent higher than those of this series.

Discussing the mode of spread of the neoplasm he found that 61 per cent of single adrenal secondary tumours were on the same side of the body as the primary tumour, 39 per cent on the other, concluding that spread to the adrenals must be lymphatic. Comparable figures for this series are 79 per cent and 21 per cent. This difference, although based on smaller numbers, is also significant. Many more of the adrenal metastases were bilateral, in all 60 per cent. In several the ipsilateral was noted to be larger in size than the contralateral, but more often than not no mention was made as to the relative dimensions.

Onuigbo (1957) found that 65 per cent of renal metastases were ipsilateral. In this material renal metastases were seen in 17 per cent, 37 per cent of which were ipsilateral, 37 per cent contralateral and the remaining 26 per cent bilateral. Brain spread was noted in 24 per cent, being ipsilateral in 39 per cent, contralateral in 32 per cent and on both sides in 29 per cent. Meyer and Reah (1953) found cerebral metastases in 25 per cent of their necropsy cases.

The figures for extrathoracic metastasis in this series are in close agreement with those of Bryson and Spencer (1951). Pleural effusion was seen in 25 per cent, two-thirds of which were blood-stained.

Trachea

There were two cases of neoplasm of the trachea. One in a 16-year old Chinese boy, which straddled the trachea at the level of the suprasternal notch, infiltrating surrounding muscle and the oesophagus, proved to be a round cell sarcoma. The other arose 5 cm below the vocal cords in a Chinese male aged 41 years, infiltrating the outer oesophagus. Histologically the tumour was of epidermoid origin. Tracheal tumours are rare: they have been well described by Culp (1938).

Larynx

There were 12 cases of laryngeal carcinoma, one in a Tamil Indian woman aged 47 years; the remainder in Chinese males, whose mean age at death was $52{\cdot}5 \pm 6{\cdot}7$ years.

17 per cent of the tumours were found on the left vocal cord, 17 per cent on the right. 25 per cent involved both cords and 25 per cent arose from the epiglottis, while 17 per cent involved both the vocal cords and the epiglottis. Lymph node involvement was noted in 50 per cent of cases, and was usually bilateral. Spread to the lung was seen in 25 per cent, to the liver in 17 per cent. The incidence of metastasis is high, spread from tumours of the endolarynx usually being rare (Ackerman and Regato, 1954). This is no doubt due to the extensive nature of most of the tumours.

In the five-year period 1954–58 neoplasms of the larynx accounted for 2·0 per cent of hospital admissions with malignant disease, 2·2 per cent of hospital cancer deaths, 0·6 per cent of post-mortems on cases of malignant disease, and 1·8 per cent of cancer deaths recorded by the Registrar-General (Singapore).

In 1940–42 1·7 per cent of all cancers recorded by the Registrar-General, England and Wales, were in the larynx (Kennaway, 1950), the ratio of larynx to lung cancers being 1 : 4·4. In Singapore for 1954–58 inclusive the ratio is 1 : 6·2.

These tumours are thus substantially as seen elsewhere and will not be discussed further.

DISCUSSION

Hoffman (1915) quoted, from the Annual Reports of the Medical Department of the Straits Settlements, figures of relative incidence for the 121 cancer cases seen at Tan Tock Seng's Hospital, Singapore, from 1907 to 1912 inclusive. 5·8 per cent of these were in the lung. If we can accept that a further 4·1 per cent described as " sarcoma of the mediastinum " may have been oat cell or undifferentiated carcinomata, the overall incidence was then 9·9 per cent.

The late Dr. J. C. Tull, sometime Senior Pathologist, Singapore, provided comparative data for a paper by Bonne (1937) on the incidence of malignant disease in South-East Asia. He found that 6·2 per cent of malignant tumours at post-mortem were in the lung. In 1948–58 inclusive, in a total of 1096 malignant tumours seen at autopsy, 176 or 15·7 per cent arose in the lung.

Prior to 1954 tumours of the larynx, lung and trachea were not separated in the returns of both the hospitals and the Registrar-General (Singapore). Since then cancers of the larynx (List No. 161) have been entered separately, but trachea is still included with lung (List No. 162–3). It is felt that the number of tracheal tumours is negligible and henceforth in this paper when the word lung is used in connection with hospital and Registrar's figures, strictly it should read lung and trachea.

The crude death rate for cancer of the lung for 1954–58 was 0·065 per 1000 living per annum. A comparable rate for England and Wales for 1951–55 was 0·355 per 1000 living per annum. As the Singapore population has such an unusual structure, more than half being under the age of 21 years, and as the age specific death rates are not at present available for individual tumours, although available by sex for neoplasms as a whole, further comparison seems pointless.

In the quinquennium 1954–58 there were 7131 admissions to hospital with some form of malignant disease (List No. 140–205). Of these tumours 10·8 per cent were diagnosed as being in the lung. 21·4 per cent of those admitted died, and of these deaths 16·4 per cent were due to lung cancers. In this period 660

post-mortems were carried out on cases of malignant disease, 15·2 per cent of which were found to be pulmonary in origin. The Registrar-General (Singapore) noted that 10·9 per cent of all deaths due to malignancy were notified as lung cancers. Cancer of the lung would thus appear to account for between 10 and 15 per cent of all malignant tumours in Singapore. Kennaway (1950) gives an abstract of deaths from malignant disease by form and site, in England and Wales for 1940–42, based on the returns of the Registrar-General : cancer of the lung accounted for 7·6 per cent of carcinomata.

City life is held to increase lung cancer morbidity (Stocks, 1959) and although a large proportion of the Singapore population lives in the city proper, the atmosphere is but little polluted by heavy industry which is, as yet, virtually non-existent. There is, however, a high density of vehicular traffic propelled by petrol and diesel engines, many of which emit excessive exhaust.

In Britain, for the period 1951–55, Doll (1958) gives the mean annual consumption of cigarette tobacco for males over the age of 14 years as 7·8 lb. (3·5 kg) ; for females as 2·6 lb (1·2 kg). The mean value for both sexes, assuming sex parity, being 5·2 lb. per person (2·4 kg). In Singapore, a comparable mean value for both sexes over the age of 14 years, based on issues from bond for consumption on the Island of both imported and locally manufactured cigarettes, for the years 1956–58 inclusive, is 6·2 lb. (2·8 kg) per person per annum. As many children under the age of 15 years appear to smoke perhaps the mean figure of 3·7 lb. (1·7 kg) of cigarette tobacco per person per annum may be of more significance. The consumption of pipe tobacco is low, 0·12 lb. (0·06 kg) per head of population.

An unknown number of persons in Singapore smoke opium, a drug to which occidentals are not usually exposed. Of these 633, all males, were sent in 1957 to the Opium Treatment Centre for rehabilitation. Whether opium has any carcinogenic effect is, of course, another matter.

Cancer of the lung is not unknown in the other countries of South-East Asia. Vellios, Goonchorn and Suvanatemiya (1953) described the tumours seen in two hospitals in Thailand. In a ten-month period 198 autopsies were performed, in which material there was but one lung cancer, and this an incidental discovery. This was the only lung tumour seen in 350 malignant neoplasms, both post-mortem and biopsy.

Piyaratn (1959) reviewing 1100 biopsies of malignant tumours (and including some of those already described by Vellios *et al.* (1953)) submitted to the Department of Pathology, Chulalongkorn Hospital, Bangkok, found 24 lung tumours, 20 of which were in males. From this evidence he states " . . . that bronchogenic carcinoma is rather common in Thailand . . . ", but does not attempt to relate his material to the Bangkok population as a considerable number of patients came from without the city.

In Ceylon, Cooray and Leslie (1958) found 5 bronchial carcinomas in the 2562 post-mortems carried out in the five years 1952–56—an incidence of 0·2 per cent. A further 22 cases were diagnosed on biopsy. The post-mortem incidence is thus somewhat lower than in Singapore, as is the consumption of tobacco.

Kouwenaar (1951) describes a post-mortem series of 1301 Chinese and 1189 Javanese males in Sumatra. Malignant disease was found in 120 and 59 respectively. Lung cancer accounted for 9 per cent of the malignancies in the Chinese, and for just under 1 per cent in the Javanese. Marsden (1958) in his survey of

4650 biopsy malignant neoplasms seen in Malaya, finds that 7·8 per cent of malignant tumours in the Chinese male, and 2·1 per cent in the Chinese female, are pulmonary. Figures for the Malay and Indian of both sexes, are respectively 2·9 and 1·1 per cent, 3·8 and 1·3 per cent. These observations are of some import, as from the figures of the author and those from Sumatra it might be assumed that lung cancer is rare in the Malay.

Shih *et al.* (1959) discuss, from the clinical aspect, 236 cases of bronchogenic carcinoma seen in Shanghai from 1949–57 inclusive. These cases were proven by biopsy or cytology ; a further 700 diagnosed by X-ray alone were not described further. The sex ratio was 3·5 : 1, males predominating. 16 per cent of patients were below the age of 40 ; considerably more than the Brompton Hospital series and slightly more than in Singapore. 55 per cent of these patients were noted to be habitual smokers.

Yeh and Cowdry (1954) examined 1869 malignant tumours collected in Taiwan (Formosa). These comprised 1729 biopsies and 140 autopsies on persons with malignant disease. A total of 19 lung cancers were encountered, 3 post-mortem, 16 removed surgically, or 1·02 per cent of all the tumours. The peak incidence of these tumours was, as in this series, in the decade 50–59. The sources of bias in this material are fully discussed.

Stransky and Felix (1951) found 1 lung cancer in 61 necropsies on persons with malignant disease performed between 1911 and 1919 at the Philippine General Hospital, Manila. Between 1945 and 1950, at the same hospital, there were 141 cases of malignancy with post-mortem examination, lung tumours comprising 4 per cent. In a total of 919 malignant tumours diagnosed on biopsy at the University of San Tomas by Sta. Cruz and de los Santos (1955) between 1946–53, 0·3 per cent were in the lung. In the 122 cases of cancer who came to post-mortem, 8·4 per cent of all autopsies, 4 (3 per cent) were pulmonary in origin.

In brief, cancer of the lung is found throughout Asia. At present its incidence at large seems to be low, although where figures are available about one-tenth of all deaths from malignant neoplasm are in the lung, a value not far removed from the reported relative incidence in the West.

SUMMARY

The main morbid anatomical features of the 176 lung, the 2 tracheal, and the 13 laryngeal cancers seen in the 22,997 post-mortems performed by the University and Government Departments of Pathology, Singapore, from 1948–58 inclusive, are described, and are seen to be as elsewhere.

About one-tenth of all cancers admitted to hospital, and of all cancers registered by the Registrar-General (Singapore) from 1954–58 inclusive were in the lung.

In 15 per cent of all post-mortems on persons with malignant disease, the primary tumour was of pulmonary origin.

The crude death rate for cancer of the lung in Singapore for 1954–58 inclusive was 0·065 per 1000 living per annum.

More cases of lung cancer are seen before the age of 40 than in the West.

The consumption of tobacco in Singapore is noted to be 3·7 lb. (1·7 kg) per person per annum.

A portion of the literature on cancer of the lung in South-East Asia and in China is discussed.

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REFERENCES

ACKERMAN, L. V. AND REGATO, J. A. DEL.—(1954) 'Cancer. Diagnosis, Treatment and Prognosis'. 2nd Edn. London (Kimpton), p. 416.
BIGNALL, J. R.—(1958) in 'Monographs on Neoplastic Disease. Vol. 1. Carcinoma of the Lung'. Edinburgh (Livingstone), p. 285.
BONNE, C.—(1937) *Amer. J. Cancer*, **30**, 435.
BRYSON, C. C. AND SPENCER, H.—(1951) *Quart. J. Med.*, **20**, 173.
COORAY, G. H. AND LESLIE, N. D. G.—(1958) *Brit. J. Cancer*, **12**, 1.
CULP, O. S.—(1938) *J. thorac. Surg.*, **7**, 471.
DOLL, R.—(1958) in 'Monographs on Neoplastic Disease. Vol. I. Carcinoma of the Lung'. Edinburgh (Livingstone), p. 71.
HOFFMAN, F. L.—(1915) 'The Mortality from Cancer throughout the World'. Newark, New Jersey (The Prudential Press), p. 712.
KENNAWAY, E. L.—(1950) *Brit. J. Cancer*, **4**, 158.
KOUWENAAR, W.—(1951) *Docum. neerl. indones. Morb. trop.*, **3**, 357.
MARSDEN, A. T. H.—(1958) *Brit. J. Cancer*, **12**, 161.
MEYER, P. C. AND REAH, T. G.—(1953) *Ibid.*, **7**, 438.
MUIR, C. S.—(1959) *Ibid.*, **13**, 595.
OCHSNER, A. AND DE BAKEY, M.—(1942) *J. thorac. Surg.*, **11**, 357.
Idem, DIXON, J. L. AND DE BAKEY, M.—(1945) *Clinics*, **3**, 1207.
ONUIGBO, W. I. B.—(1957) *Brit. J. Cancer*, **11**, 175.
PIYARATN, P.—(1959) *Cancer*, **12**, 693.
SHIH MEI-HSIN, KU SUEI-YÜEH, CHANG CHIÜ-PING, KU K'AI-SHIH, TIAO YU-TAO AND CHU ERH-MEI.—(1959) *Chin. med. J.*, **79**, 19.
STA. CRUZ, J. Z. AND DE LOS SANTOS, R.—(1955) *J. Phil. Is. med. Ass.*, **31**, 637.
STOCKS, P.—(1959) *Practitioner*, **182**, 667.
STRANSKY, E. AND FELIX, N. S.—(1951) *J. Phil. Is. med. Ass.*, **27**, 106.
VELLIOS, F., GOONCHORN, S. G. AND SUVANATEMIYA, P.—(1953) *Cancer*, **6**, 188.
YEH, SHU AND COWDRY, E. V.—(1954) *Ibid.*, **7**, 425.

EPIDEMIOLOGICAL EVIDENCE FROM CHEMICAL AND SPECTROGRAPHIC ANALYSES THAT SOIL IS CONCERNED IN THE CAUSATION OF CANCER

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THE reasons which led to the initiation in 1952 of a study of the chemical properties of garden soils in connection with cancer in North Wales and Cheshire have been detailed (Stocks, 1957, 1958). The Cheshire and North Wales Branch of the Campaign had included soil amongst the environmental items to be investigated in the 4-year cancer survey, and with their help the Department of Agricultural Chemistry was enabled to undertake the chemical analyses, and at a later date spectrographic estimations of trace elements. Collection of soil samples from gardens of houses where a death of a resident had occurred from cancer or other cause was made possible by the co-operation of the Medical Officers of Health and was carried out by their Public Health Inspectors.

Since 1957 the laboratory work on some 2,000 soils has continued with the aid of a grant to the University College of North Wales, a special study has been made also of two districts with high and low incidence of stomach cancer in Devonshire, and analytical work on plants and spectroscopic study of polycyclic hydrocarbons have been started. Brief progress reports have appeared in the Annual Reports of the British Empire Cancer Campaign for 1955 to 1958, and the purpose of this paper is to present a more complete and up-to-date account of the outcome so far of the work on soils, including the Devonshire study.

A hypothesis that one of the causes of cancer of the stomach is in some way connected with soil cannot be dismissed on the grounds that we do not know how such a connection could arise. Kant once remarked that " Philosophy is often much embarrassed when she encounters certain facts which she dare not doubt yet will not believe for fear of ridicule ". The soil hypothesis has met with scepticism from those who look for a single " cause " of cancer, but as Sir Julian Huxley (1958) wrote in discussing the statistical evidence about lung cancer, " It is surely time that we should drop mediaeval concepts concerning causation and think in terms of multiple correlation ". Having established as a first step the existence of statistical correlations between the incidence of stomach cancer and the amounts of organic material and certain trace elements in the neighbouring soil. possible reasons for this such as vegetables, water supply, air pollution and radioactivity have next to be investigated.

Organic Content of Soil in Districts having Different Rates of Stomach Cancer Mortality

In the North Wales and Chesire region where soils contain inconsiderable amounts of free calcium carbonate the loss of weight on ignition of a dry soil, expressed as a percentage, provided an index of the organic matter present. Where the soils are contaminated with coal or ashes the loss on ignition would be

affected, so a second index was obtained of the readily oxidisable or "organic carbon" by a wet chemical oxidation of the soil (Walkley and Black, 1934). In this method, coal and ash are but little affected and so do not contribute to the index. Soils seen on examination in the dried state to be appreciably contaminated by small coal have, however, been dealt with separately in the statistical analysis.

During the years whilst soil samples were being collected the aim was to obtain a sample from every house where a death occurred from cancer of the stomach, intestine, rectum, larynx, lung or breast or from leukaemia or Hodgkin's disease, and in the counties of Anglesey, Caernarvon and Cheshire omissions arose only through absence of a garden or inability to contact relatives or obtain the necessary permission. In the other counties the coverage was not so complete owing to pressure of work on the Health Officers, but such selection of cases as resulted was connected with periods of time and not with the nature of the cases. Histories of all persons dying of cancer were obtained independently from relatives if no hospital history had been obtained previously, and this information included the duration of residence at the house prior to death. In order to obtain a control series addresses were taken from the monthly lists of deaths from causes other than cancer by matching the sex, age and district of residence with the first 600 stomach cancer cases as nearly as was practicable.

The soil study covered the 55 administrative areas in North Wales and 11 in the parts of Cheshire within the Liverpool Hospital Region, excluding the county boroughs, and details of these were given by Stocks (1957, Table 6 and Map 2).

The standardised mortality ratios (S.M.R.'s) for cancer of the stomach in the 8-year period 1947–55 were given in the final column of that table and depicted in Map 3, and they ranged from 65 to 262 compared with 100 for England and Wales, four very small districts with fewer than 5 deaths being combined with adjoining areas. On page 104 of the report a general similarity between the geographical distribution of stomach cancer mortality and organic carbon content of soils not directly associated with cases of cancer was pointed out, whereas no such correspondence was apparent with cancer mortality rates for intestine, lung or breast.

When the districts are divided up into 9 groups with stomach cancer S.M.R. 50–, 75–, 100–, 125–, 150–, 175–, 200–, 225–, 250–274, and soils distributed on scales of organic carbon and ignition loss, the correlation coefficients between organic content and the mortality from stomach cancer in the district where the soil was taken are as shown in Table I.

TABLE I.—*Correlations Between Measures of Organic Matter Content of Soils not Directly Connected with a Case of Stomach Cancer and the Level of Mortality from Stomach Cancer in the District from which the Soil was taken, Estimated from Two Alternative Series of Samples*

	(1) Non-cancer control soils			(2) Non-gastric cancer soils			Correlation with S.M.R. from stomach cancer in	
	Number	Mean	S.D.	Number	Mean	S.D.	1st Series	2nd series
Organic carbon	513	2·98	1·22	1063	2·86	1·35	0·4661	0·4015
Ignition loss*	373	9·34	4·12	681	9·24	4·09	0·5693	0·5027

* When the ash-contaminated soils are included the coefficients become 0·481 in the non-cancer series and 0·462 in the other series.

TABLE II.—*Organic Carbon Content of Soils from Houses with Stomach Cancer, showing Excess over the Calculated Frequencies based on the Corresponding Distributions of Other Cancer and Non-cancer Cases (corrected for the Relative Mortality from Stomach Cancer in the District).*

Years in house	Organic carbon per 100	No. of soils grouped according to S.M.R. of district							Sub-totals		Excess as calculated from			
											Non-	Other	Non-	Other
		50–	100–	125–	150–	175–	200+	All	50–	150+	50–	50–	150+	150+
Under 10	0–	18	18	7	3	3	2	51	43	8	7·1	6·5	1·8	−1·4
	2–	12	19	15	18	5	12	81	46	35	−12·1	−5·6	15·6	21·4
	3·5–	4	4	7	8	6	4	33	15	18	4·8	−1·0	8·5	7·3
	5+	—	1	4	5	2	4	16	5	11	3·0	3·4	6·8	5·6
	Total	34	42	33	34	16	22	181	109	72	2·8	3·3	32·7	32·9
10–19	0–	11	8	7	5	2	2	35	26	9	1·1	−6·6	6·1	3·1
	2–	19	19	8	16	3	6	71	46	25	4·5	3·0	3·5	10·6
	3·5–	2	3	4	8	6	6	29	9	20	−9·0	−0·4	9·2	5·5
	5+	—	5	4	5	4	9	27	9	18	4·0	4·7	15·2	14·1
	Total	32	35	23	34	15	23	162	90	72	0·6	0·7	34·0	33·3
20 and over	0–	13	8	8	2	—	—	31	29	2	13·0	−8·8	−4·2	−6·6
	2–	16	21	19	37	16	17	126	56	70	−18·6	4·4	42·5	44·4
	3·5–	5	11	14	26	13	13	82	30	52	0·7	2·0	9·0	18·6
	5+	4	4	9	10	4	11	42	17	25	13·0	8·0	17·1	10·2
	Total	38	44	50	75	33	41	281	132	149	8·1	5·6	64·4	66·6

Years in house	Organic carbon per 100	Organic carbon distributions of soils from houses in same groups of districts where a death had ocurred from: Non-cancer cause							Other cancer (i.e. not stomach)						
		50–	100–	125–	150–	175–	200+	All	50–	100–	125–	150–	175–	200+	All
Under 10	0–	6	6	9	4	2	2	29	39	33	10	12	6	2	102
	2–	11	9	9	14	10	4	57	36	45	33	16	8	18	156
	3·5–	1	2	2	6	5	6	22	7	13	18	15	7	13	73
	5+	1	—	—	3	2	1	7	1	—	3	5	2	10	21
	Total	19	17	20	27	19	13	115	83	91	64	48	23	43	352
10–19	0–	10	1	2	3	—	—	16	40	17	8	5	3	2	75
	2–	12	2	10	10	10	6	50	33	32	22	16	9	9	121
	3·5–	4	1	2	8	3	2	21	4	6	11	12	7	3	43
	5+	—	1	—	2	—	1	4	3	1	6	3	1	4	18
	Total	26	6	14	23	13	9	91	80	56	47	36	20	18	257
20 and over	0–	9	2	2	1	2	3	19	32	21	13	4	7	3	80
	2–	18	11	14	20	8	4	75	34	32	28	27	7	10	138
	3·5–	4	6	11	33	10	10	74	10	11	24	37	15	5	102
	5+	—	—	3	5	3	2	13	3	5	8	11	2	11	40
	Total	31	19	30	59	23	19	181	79	69	73	79	31	29	360

TABLE III.—*Loss on Ignition of Dried Soils from Houses with Stomach Cancer, Showing Excess over the Calculated Frequencies based on the corresponding Distributions of Other Cancer and Non-cancer Cases (Corrected for the Relative Mortality from Stomach Cancer in the District)*

Years in house	Loss on ignition per 100	No. of soils grouped according to S.M.R. of district							Sub-totals		Excess as calculated from			
		50–	100–	125–	150–	175–	200+	All	50–	150+	Non- 50–	Other 50–	Non- 150+	Other 150+
Under 10	0–	24	25	11	8	3	3	74	60	14	−6·7	0·6	0·7	5·2
	8·5–	7	12	7	9	3	3	41	26	15	0·1	−0·6	6·6	2·8
	11·5–	4	2	8	7	4	3	28	14	14	3·0	0·1	5·4	6·4
	14·5+	2	3	10	10	6	13	44	15	29	6·4	3·1	19·9	16·6
	Total	37	42	36	34	16	22	187	115	72	2·8	3·2	32·6	31·3
10–19	0–	23	20	9	11	2	6	71	52	19	−2·1	2·4	13·3	12·3
	8·5–	6	9	3	9	1	5	33	18	15	−2·1	−7·2	−6·7	4·9
	11·5–	4	5	3	5	5	4	26	12	14	3·8	3·8	2·0	3·3
	14·5+	1	4	8	10	8	8	39	13	26	0·7	1·5	11·6	13·2
	Total	34	38	23	35	16	23	169	95	74	0·3	0·5	33·6	33·7
20 and over	0–	19	8	11	9	2	3	52	38	14	−14·2	−16·9	6·7	5·7
	8·5–	7	15	10	17	6	7	62	32	30	−0·4	6·9	11·9	13·9
	11·5–	7	11	11	22	12	19	82	29	53	8·9	24·1	31·6	37·9
	14·5+	5	6	16	29	13	12	81	27	54	7·8	1·5	17·3	10·1
	Total	38	40	48	77	33	41	277	126	151	2·1	5·6	67·5	67·5

		Ignition loss distributions of soils from houses in same groups of districts where a death had occurred from: Non-cancer cause							Other cancer (i.e. not stomach)						
		50–	100–	125–	150–	175–	200+	All	50–	100–	125–	150–	175–	200+	All
Under 10	0–	19	15	11	10	3	5	63	59	41	17	9	5	6	137
	8·5–	10	2	5	7	3	2	29	10	25	21	12	9	6	83
	11·5–	2	2	3	6	6	2	21	5	14	11	7	4	11	52
	14·5+	2	2	1	5	7	2	19	6	4	14	20	5	21	70
	Total	33	21	20	28	19	11	132	80	84	63	48	23	44	342
10–19	0–	22	9	6	5	1	—	43	53	23	8	4	4	3	95
	8·5–	5	3	4	4	3	2	21	14	18	14	10	7	3	66
	11·5–	4	—	3	4	6	4	21	2	5	13	10	4	5	39
	14·5+	1	2	3	8	3	3	20	4	6	15	13	7	8	53
	Total	32	14	16	21	13	9	105	73	52	50	37	22	19	201
20 and over	0–	34	10	6	4	3	3	60	55	34	16	4	5	5	119
	8·5–	8	10	10	15	4	3	50	14	19	12	15	5	7	72
	11·5–	5	4	5	15	4	5	38	5	8	12	14	6	5	50
	14·5+	6	2	8	25	11	8	60	8	6	29	47	13	11	114
	Total	53	26	29	59	22	19	208	82	67	69	80	29	28	355

There is an unmistakable relation between the average amount of organic material in the soil samples taken throughout a district and the mortality there from stomach cancer, but the regression of the latter upon the former is not a simple one. As shown below, the association between the factors is very pronounced in the part of the scale where the index lies between 2 and 3¼ :—

Organic carbon index	.	.	.	1¼	2¼	3¼	4¼	5¼
Mean stomach cancer S.M.R.	.	.		116	123	160	170	181
Increment of S.M.R. per unit	.	.			7	37	10	11

This accords with the finding in the next section that stomach cancer cases occurred with peculiar frequency after long residence in houses where the soil contained between 2·0 and 3·5 parts per 100 of organic carbon. Soils of the "β" group described by Davies and Wynne Griffith (1954) and found by them to be associated significantly with stomach cancer deaths in Anglesey also tend to have an organic carbon content about this magnitude.

Organic Content of Garden Soils Directly Associated with Cancer

Soil samples were obtained from gardens of houses where a death from stomach cancer had occurred, and 690 of these have been analysed for organic carbon and 700 for loss on ignition. Information as to length of residence at the house before onset of the illness was available for 92 per cent of these and Tables II and III show the distributions on scales of organic carbon and ignition loss for durations of under 10, 10–19 and 20 or more years, with subdivision of the districts into 6 groups according to their stomach cancer mortality in 1947–54. The lower parts of the tables show the corresponding distributions of alternative control series of soils taken at houses where a death had occurred (1) from a non-malignant cause and (2) from non-gastric cancer. In calculating the expected frequencies for comparison with the stomach cancer numbers the soils were further sub-divided into those with and without ash contamination, each being calculated separately. The aggregated result for each district group was then divided by the stomach cancer mortality ratio to obtain the expected frequency distribution if stomach cancer incidence were uniform and unrelated to soil characters. The divergencies from expectation in the right hand columns of the upper parts of the tables show, therefore, how the stomach cancer cases *in excess of the normal* were distributed on the soil scales, the "normal" being derived in two ways, firstly from a matched control series where cancer was not known to have occurred and secondly from the unselected series associated with cancers of the intestine, lung, breast and a few other sites.

In districts where the incidence of stomach cancer is below or or not greatly above the national average (S.M.R. 50–149) a condensed comparison between the actual and expected distributions of soils from houses where stomach cancer occurred is shown in Table IV.

The only differences noticeable here are a concentration of the stomach cases on soils with organic carbon exceeding 5 (31 compared with a mean expectation of 12), and a tendency for the stomach cases to occur where the soil has an ignition loss of 11·5 or more (110 compared with a mean expectation of 82·5). It is evident that in the districts with not very abnormal stomach cancer incidence there is a tendency for high content of organic material in the garden soil to be associated with occurrence of stomach cancer in the house.

TABLE IV.—*Actual and Expected Organic Content of Garden Soils Associated with Stomach Cancer in Districts with S.M.R. Below* 150

	Organic carbon				Ignition loss (per cent)				
	0–	3·5–	5+	Total	0–	8·5–	11·5–	14·5+	Total
Resident less than 20 years in house									
Actual cases	161	24	14	199	112	44	26	28	210
Expected from Non-cancer	160·4	28·2	7·0	195·6	120·8	46·0	19·2	20·9	206·9
Expected from Other cancer	163·7	25·4	5·9	195·0	109·0	51·8	22·1	23·4	206·3
Resident 20 years or more in house									
Actual cases	85	30	17	132	38	32	29	27	126
Expected from Non-cancer	90·6	29·3	4·0	123·9	52·2	32·4	20·1	19·2	123·9
Expected from Other cancer	89·4	28·0	9·0	126·4	54·9	25·1	14·9	25·5	120·4

In the aggregate of districts where mortality from stomach cancer is very high (S.M.R. 150–262), if the large excess of cases over the normal expectation is unconnected with soil characters its distribution on the soil scales should not differ significantly from the distribution of the expected cases, and if there is a concentration of the excess at a particular point in the soil scale that would support the hypothesis that a soil factor accounts for the abnormally high incidence of stomach cancer in parts of North Wales, this being superimposed on the other causes which operate throughout England and Wales. The comparison summarised in Table V shows amongst people who had lived less than 20 years in the house a strong concentration of the stomach cancer cases on soils with high organic carbon and ignition loss, similar to that found in the more normal districts (Table IV). For people who had lived 20 years or more in the house and then died of cancer or other cause, the surplus of stomach cancer is distributed

TABLE V.—*Actual and Expected Organic Content of Garden Soils Associated with Stomach Cancer in Districts with High S.M.R.* (150+)

	Organic carbon					Ignition loss (per cent)				
	0–	2–	3·5–	5+	Total	0–	8·5–	11·5–	14·5+	Total
Resident less than 20 years in house										
Actual cases	17	60	38	29	144	33	30	28	55	146
Expected from Non-cancer	9·1	40·9	20·3	7·0	77·3	19·2	16·7	20·6	23·3	79·8
Expected from Other cancer	15·3	28·0	25·2	9·3	77·8	15·2	22·3	18·3	25·2	81·0
Excess (per cent) over mean expectation	39	74	67	256	86	92	54	44	127	82
Per cent of total										
Mean expected number	15·7	44·4	29·4	10·5	100·0	21·4	24·2	24·2	30·2	100·0
Excess cases	7·2	38·4	23·0	31·4	100·0	24·1	16·0	13·0	46·9	100·0
Resident 20 years or more in house										
Actual cases	2	70	52	25	149	14	30	53	54	151
Expected Non-cancer	6·2	27·5	43·0	7·9	84·6	7·3	18·1	21·4	36·7	83·5
Expected Other cancer	7·4	26·6	38·2	11·3	83·5	8·3	16·1	15·2	43·9	83·5
Excess (per cent) over mean expectation	−73	164	36	120	78	79	70	190	34	81
Per cent of total										
Mean expected number	8·1	32·2	48·3	11·4	100·0	9·3	20·5	21·9	48·3	100·0
Excess cases	−7·4	66·1	17·6	23·7	100·0	9·2	19·1	51·4	20·3	100·0

on the soil scales in a manner very different from that expected. Two thirds of the excess occurs on soils with organic carbon between 2 and 3·5 per 100 compared with one third of the control, and one half occur on soils with ignition loss between 11·5 and 14·5 per cent compared with about one fifth of the control. These differences from expectation are highly significant from a probability standpoint, and they seem to indicate that long residence on soil of this special type in North Wales is peculiarly conducive to cancer of the stomach, the connection with soils of high organic content being more pronounced where there was appearance of stomach cancer after shorter periods of residence.

In Table VI(*b*) the mean organic carbon and ignition loss of soils from 43 houses where a death from stomach cancer (SC) had occurred in two Devonshire townships are compared with a control series taken from corresponding subdistricts of those areas The SC series gives higher mean values than the control. (N) both for organic carbon and for ignition loss. Furthermore, as shown in Table VI(*a*), the general level of organic matter in the soil is higher in the locality with a high death rate from stomach cancer than in the locality with a low rate. These results, obtained by a more rigid control method than was possible in North Wales, agree with what has been found there.

Trace Elements in Garden Soils in North Wales, Cheshire and Devonshire Districts with High and Low incidence of Stomach Cancer

Two neighbouring and similar Devonshire townships A and B each with about 4,000 people, have shown over the last 10 years very different death rates from cancer of the stomach, the S.M.R's being estimated at 187 and 52 respectively. Owing to dissemination at A of industrial waste containing mineral substances which could be carcinogenic, a comparison of garden soils in A and B was carried out with the co-operation of the County Health Officers in respect of the trace element contents, using the spectrographic method. Each place was divided into 24 sub-areas and in each of these three gardens were chosen at random, except that addresses where cancer was known to have occurred were avoided, soil samples being taken and mixed together producing 24 samples representative of the sub-areas in A, and likewise in B. The dried soils were analysed spectrographically for 7 elements and chemically for iron and copper by the methods described on page 20. The mean values in parts per million for the two places are shown in Table VI(*a*).

The average amounts of 7 of the elements were greater in the soils from A where the stomach cancer mortality is high than in those from B where it is low, the excess being highly significant for cobalt, nickel and iron. Neither in district A nor in B are the values necessarily representative of Devonshire soils in general, and no useful comparison with the averages for North Wales and Cheshire in Table VII can be made.

In the process of obtaining a control series of garden soils for trace element analysis 36 from parts of North Wales where the incidence of stomach cancer is abnormally high and 48 from Cheshire where it is normal were chosen from the addresses where a person had died of a cause other than cancer after 10 years or more of residence in the house. Comparison of the results of spectrographic analysis in respect of 8 elements is shown in Table VII, the method used being the same as for the Devon soils (p. 20).

TABLE VI.—*Trace Elements and Organic Matter in Garden Soils of Two Devonshire Townships*

(*a*) Comparison between soils randomly taken from A where mortality from stomach cancer is high and from B where the mortality is low.

	Number in each place	Mean content in soils from A	Mean content in soils from B	Difference A − B	Per cent of combined average
Organic carbon (per cent)	72	3·22	2·82	0·40	13
Ignition loss (per cent)	72	11·07	7·90	3·17	29
Copper (parts per million)	72	2·62	1·95	0·67	25
Zinc " " "	72	115	120	−5	..
Chromium " " "	72	0·215	0·192	0·023	11
Cobalt " " "	72	0·462	0·326	0·136	35
Nickel " " "	72	1·68	1·17	0·51	36
Vanadium " " "	72	0·286	0·362	−0·076	..
Titanium " " "	72	0·190	0·153	0·037	22
Iron " " "	72	24·9	17·7	7·2	34
Lead (median values in p.p.m.)	72	3·7	2·9	0·8	24

(*b*) Comparison between soils from houses where stomach cancer had occurred (SC) and a control series from the same sub-districts of A and B (N).

	Number in each group	Mean content in soils of series SC	Mean content in soils of series N	Difference SC − N	Ratio to standard error
Organic carbon (per cent)	43	3·46	3·02	0·44	1·4
Ignition loss (per cent)	43	11·90	9·96	1·94	1·7
Copper (parts per million)	44	2·68	2·18	0·50	1·5
Zinc " " "	44	181	103	78	2·8
Chromium " " "	44	0·289	0·193	0·096	2·8
Cobalt " " "	44	0·502	0·371	0·131	2·5
Nickel " " "	44	1·93	1·48	0·45	2·2
Vanadium " " "	44	0·407	0·303	0·104	2·0
Titanium " " "	44	0·132	0·170	−0·038	..
Iron " " "	44	19·5	21·8	−2·3	..
Lead (median values in p.p.m.)	44	2·05	3·50	−1·45	..

TABLE VII.—*Trace Element Content of Garden Soils not Directly Connected with Cancer in North Wales and Cheshire*

	Mean content (p.p.m.) in North Wales (36)	Mean content (p.p.m.) in Cheshire (48)	Difference	Ratio to its standard error
Zinc	51·2	54·3	−3·1	0·2
Chromium	0·230	0·178	+0·052	2·0
Cobalt	0·522	0·528	−0·006	0·1
Nickel	1·046	1·230	−0·184	1·8
Titanium (soluble)	0·228	0·213	+0·015	0·7
Vanadium	0·342	0·455	−0·113	2·1
Iron	23·8	20·7	+3·1	1·2
Lead (median values)	2·1	5·2	−3·1	..

The North Wales soils show an excess of chromium, whilst nickel, vanadium and lead are more plentiful in the Cheshire soils. There is no indication from these comparisons that the general level of zinc is any greater in districts with high incidence of cancer of the stomach than it is elsewhere, and for cobalt this was the case in the Devonshire townships investigated but not in the Cheshire and North Wales area. This has to be kept in mind when seeking an explantion of the results recorded in the next section. The excess of chromium, cobalt and nickel in locality A (Table VI*a*) may be of industrial origin and investigation of this possibility is not yet complete.

Trace Elements in Garden Soils Directly Associated with Cancer

In the Devonshire township A, already referred to, 37 deaths from cancer of the stomach had occurred in 15 years, and soil samples were taken from the gardens of houses where the deceased persons had last lived, the number of years residence there before death being ascertained when possible. Within township B 7 such deaths had occurred and samples were taken similarly. Each of the 44 cases was matched by a non-cancer sample from the same sub-area, and Table VI(*b*) compares the mean trace element contents of the soils in the stomach cancer (SC) and non-cancer (N) series.

In North Wales spectrographic examination has been made of 73 soils from houses in the 5 counties where a resident had died of stomach cancer after living there 10 years or more, and of 39 soils similarly associated with a death from cancer of the intestine, lung or breast, the frequencies of these being 29, 7 and 3 respectively. From the Cheshire areas, comprising the Wirral, Runcorn, Lymm and the rural districts of Chester and Tarvin, 31 soils associated with stomach cancer and 9 associated with intestinal cancer have been analysed. The mean trace element contents of these groups (SC and OC) are compared in Table VIII with those for the non-cancer control series (N).

Zinc.—In North Wales the stomach cancer soils show a wide range of values from 4 to 441 part per million, with a mean of 81·2 compared with 51·2 for the non-cancer controls, the excess of 30·0 being twice its standard error. In Cheshire the SC soils show a range from 3 to 387 with a mean of 83·4 compared with 54·3 for the controls, the excess of 29·1 being $1\frac{1}{2}$ times its standard error. In the combined area the excess of 28·8 is 2·4 times its standard error. In the two Devon localities where the zinc levels happen to be higher the mean for the SC soils is 181 compared with 103 for the matched controls, giving an excess 2·8 times its standard error.

The odds against finding such agreement in three independent series by chance, with t = 2·0, 1·5 and 2·8, are enormously great, and it must be concluded that a zinc content of the soil higher than the local average is a factor favourable to the appearance of stomach cancer, and that this is not confined to districts where the general incidence is specially high. Indeed, as shown in Tables VI and VII, there is no tendency for districts with high stomach cancer mortality to have higher zinc levels in the soils as a whole than districts with low mortality, and yet in the gardens of houses where deaths from stomach cancer occurred the zinc level is higher than in other gardens of the same area. This seems to indicate that another factor must be present which acts in conjunction with zinc and which is more plentiful in soils of districts where stomach cancer incidence is high than

TABLE VIII.—*Quantities of Trace Elements (parts per million) in Soils from Gardens of Houses in North Wales and Cheshire where a Death had Occurred from Cancer of the Stomach (SC), Other Cancer (OC) or from a Non-cancer cause (N)*

		North Wales*			Cheshire*			Combined area*		
		No.	Mean (p.p.m.)	Diff. (C − N)	No.	Mean (p.p.m.)	Diff. (C − N)	Mean (p.p.m.)	Diff. (C − N)	S.E.
Zinc	N	36	51·2	..	48	54·3	..	53·0	..	..
	SC	73	81·2	+30·0	31	83·4	+29·1	81·8	+28·8	12·0
	OC	39	64·9	+13·7	9	56·1	+1·8	63·2	+10·2	11·8
Cobalt	N	36	0·522	..	48	0·528	..	0·525	..	..
	SC	73	0·655	+0·133	31	0·625	+0·097	0·646	+0·121	0·049
	OC	39	0·583	+0·061	9	0·522	−0·006	0·572	+0·047	..
Nickel	N	36	1·046	..	48	1·230	..	1·151	..	..
	SC	73	1·016	−0·030	31	1·412	+0·182	1·134	−0·017	..
	OC	39	0·921	−0·125	9	1·381	+0·151	1·007	−0·144	..
Chromium	N	36	0·230	..	48	0·178	..	0·200	..	..
	SC	73	0·315	+0·085	31	0·232	+0·054	0·291	+0·091	0·020
	OC	39	0·290	+0·060	9	0·154	−0·024	0·265	+0·065	0·026
Vanadium	N	36	0·342	..	48	0·455	..	0·407	..	..
	SC	73	0·356	+0·014	31	0·453	−0·002	0·385	−0·022	0·097
	OC	39	0·366	+0·024	9	0·431	−0·024	0·378	−0·029	..
Titanium	N	36	0·228	..	48	0·212	..	0·219	..	..
	SC	73	0·202	−0·016	31	0·258	+0·045	0·219	Nil	..
	OC	39	0·238	+0·010	9	0·162	−0·051	0·224	+0·005	..
Iron	N	36	23·8	..	43	20·7	..	22·1	..	..
	SC	73	21·2	−2·6	31	29·5	+8·8	23·7	+1·6	..
	OC	39	24·3	+0·5	9	21·9	+1·2	23·8	+1·7	..

		Over 5 p.p.m.			Over 5 p.p.m.			% over 5 p.p.m.		
		No.	No.	%	No.	No.	%	Median	%	C − N
Lead	N	36	12	33·3	48	25	52·1	4·75	44·0	..
	SC	73	31	42·5	31	18	58·1	4·65	47·1	+3·1
	OC	39	19	51·3	9	7	77·8	5·00	59·2	+15·2

* Garden soils where a death had occurred after 10 years of more of residence at the house.
" Diff." means the difference between the mean for the cancer group (C) and that for the control (N) group. " S.E." = standard error.

where it is low. In the Devonshire locality A this might be present in the industrial waste which has found its way in the past into many of the gardens but which would only become important in regard to stomach cancer where there was also a high zinc level, and study of this problem is continuing and will be reported upon in another paper.

In the Cheshire and North Wales region, amongst the soils associated with stomach cancer those taken from ground where vegetables or fruit were being grown showed a zinc distribution somewhat different from other garden soils, as shown in Table IX, whereas no such difference appears in the control series. The organic carbon content failed to show any differences between vegetable garden and other soil. Since zinc is an active part of some enzyme systems and is

TABLE IX.—*Zinc and Cobalt Content of Soils from Vegetable or Fruit Growing Ground Compared with Other Garden Soils*

Series	Part of garden	Zinc						Cobalt					
		0–	20–	50–	80–	140+	Total	0·1–	0·4–	0·60–	0·8+		Total
Stomach cancer	V or F	10	19	14	8	5	56	16	20	14	6		56
	Other	3	8	5	4	13	33	7	10	5	11		33
	Exp.*	5·9	11·2	8·2	4·7	3·0	33	9·4	11·8	8·2	3·6		33
		$\chi^2 = 14{\cdot}8,\ n = 3,\ P < 0{\cdot}01$						$\chi^2 = 2{\cdot}38,\ n = 2,\ P = 0{\cdot}3$					
Control	V or F	12	16	9	3	6	46	16	16	9	5		46
	Other	9	12	5	6	4	36	12	9	11	4		36
	Exp.*	9·4	12·5	7·1	2·3	6·7	36	12·5	12·5	7·1	3·9		36
		$\chi^2 = 0{\cdot}77,\ n = 3,\ P > 0{\cdot}8$						$\chi^2 = 2{\cdot}45,\ n = 2,\ P = 0{\cdot}3$					

* Distribution expected from that of the V–F series.

concerned with plant life and the processes of gastric digestion a connection between stomach cancer incidence and the amount present in soil is by no means wildly improbable.

Table VIII shows that in North Wales the average zinc content of 39 soils associated with other cancer is rather greater than the control figure, but for the 29 intestinal cases included the mean is 53·0, differing inappreciably from the control, and this is true also of the Cheshire cases which are all intestinal, so the excess is confined to the 7 lung cancer cases and is not statistically significant.

Cobalt.—In North Wales the SC soils show a range from 0·17 to 2·80 parts per million with mean value 0·655, and the N soils show a range from 0·18 to 1·04 with mean 0·522, the stomach cancer excess being twice its standard error. In Cheshire the SC range is from 0·21 to 1·55 with mean 0·625, compared with the control mean 0·528, an excess of 0·097 ($t = 1{\cdot}1$). The combined area gives an excess of 0·121 and the Devonshire data an excess of 0·131, each of these being $2\frac{1}{2}$ times their standard errors. The odds against such a result arising by chance are very great, and it must be concluded that a high cobalt level in the soil is favourable to a higher incidence of cancer of the stomach but in view of Table VIII, this seems to depend upon conjunction with some other substance, as for zinc. The other cancer series shows no significant difference from the control. Table IX shows no significant differences between the cobalt content of vegetable garden and other garden soils. The element is known, however, to be concerned in plant and animal economy and also to have carcinogenic properties, so the statistical connection between a high soil content and stomach cancer incidence is deserving of further study.

Nickel.—In North Wales the SC soils have contents ranging from 0·30 to 2·62 parts per million, those from Merionethshire having specially high values, and the mean of 1·016 is slightly less than that of the controls whose range is from 0·41 to 2·48. Amongst Cheshire SC soils only 29 per cent have values below 1 compared with 58 per cent in the Welsh series and the mean 1·412 exceeds the control figure by 0·182 ($t = 1{\cdot}6$). In the Devonshire localities the nickel levels were still higher and the SC mean exceeds the N mean by 0·45 ($t = 2{\cdot}2$). It is doubtful whether these differences indicate a connection with stomach cancer since the correlation is if anything negative in the combined Cheshire-North Wales region. Comparison between the OC and N soils shows a similar

discrepancy between the two parts of this region, neither of the differences being statistically significant.

Chromium.—The variation in content is smaller for this element, the range in SC soils being from 0·06 to 0·92 and in the controls from 0·05 to 0·71. In North Wales the SC mean of 0·315 exceeds the control mean by 0·085 which is highly significant ($t = 3·1$); and in Cheshire there is likewise an excess of 0·054 ($t = 1·5$). In the combined area the stomach cancer excess is 4 times its standard error and the Devon comparison gives a similar excess of 0·096 ($t = 2·8$). When median chromium values based on more than one S.C. and control soils were compared in 17 separate districts, the S.C. median exceeded the control median in 11; but as Table X shows, the surplus incidence occurs not at the highest chromium levels but where the content lies between 0·3 and 0·6 parts per million (33 out of 89 instead of 5·5 expected).

The North Wales soils show also for the OC series, which are mostly intestinal cancer, a mean value which is 0·060 above that of the controls and in the combined area the excess of 0·065 is statistically significant. In Table X they show a concentration between 0·3 and 0·6 and for both the stomach and other cancer soils the χ^2 test gives $P < 0·001$. The association with cancer seems to differ from that of zinc and cobalt in that (1) it applies to intestinal as well as to stomach cancer and (2) since chromium levels tend to be higher in *all* soils in districts where the incidence of stomach cancer is high it is not necessary to assume that chromium acts only in conjunction with some other substance.

TABLE X.—*Chromium Content of Garden Soils Directly Associated with Cancer Compared with Controls*

Series	Stomach cancer S.M.R.	Chromium (parts per million) 0–	0·2–	0·3–	0·4–	0·6+	Total
Non-cancer	Under 150	23	24	3	..	..	50
	150 and over	19	8	1	1	3	32
Stomach cancer	Under 150	13	6	11	6	..	36
	150 and over	14	17	10	6	6	53
	Total	27	23	21	12	6	89
	Expected*	48·0	30·5	3·8	1·7	5·0	89
Other cancer	Under 150	9	5	1	1	..	16
	150 and over	7	11	8	4	2	32
	Total	16	16	9	5	2	48
	Expected*	26·4	15·6	2·0	1·0	3·0	48

* Expected from the control, given the same weighting according to S.M.R. of district.

Vanadium.—The Devonshire data show a significant excess of 0·104 ($t = 2·0$) when the mean vanadium content of the SC series of soils is compared with the matched control, but indications from the other areas are not clear. The means are greatly affected by occasional soils with very high amounts of the element, for example in North Wales the N series includes one with 6·20 p.p.m., the next highest value being 0·80, and in Cheshire N soils the highest values were 3·44 followed by 0·97. Table XI, which avoids this difficulty, reveals a great difference between the distributions of N soils in the groups of districts with high and low stomach

cancer mortality, two thirds of the soils in the former group having less than 0·2 p.p.m. compared with one eighth in the latter. When the SC soils are compared with the distribution expected from controls within the same district groups the difference is hardly significant ($\chi^2 = 9{\cdot}1$, n = 4, P = 0·06), and a difference of the same kind is seen for other cancer, namely an excess of soils with content around 0·3 parts per million. No definite conclusions can be drawn, but there is a curious resemblance to the chromium comparisons in Table X.

TABLE XI.—*Vanadium Content of Garden Soils Directly Associated with Cancer, Compared with Controls*

Series	Stomach cancer S.M.R.	Vanadium (parts per million) 0–	0·2–	0·4–	0·6–	0·8+	Total
Non-cancer	Under 150	6	15	19	6	4	50
	150 and over	21	6	2	1	2	32
Stomach cancer	Under 150	7	14	5	6	4	36
	150 and over	26	15	5	2	5	53
	Total	33	29	10	8	9	89
	Expected*	39·1	20·7	17·0	6·0	6·2	89
Other cancer	Under 150	4	6	4	—	2	16
	150 and over	11	14	2	1	4	32
	Total	15	20	6	1	6	48
	Expected*	22·9	10·8	8·1	2·9	3·3	48

* Expected from the control, given the same weighting according to S.M.R. of district.

Titanium.—The analyses relate to the titanium extractable by the standard solvent used in preparing the soil for spectrographic study, the insoluble forms such as rutile which are plentiful in soil not being thought likely to have any biological activity. In none of the areas is there any indication of any connection with cancer.

Iron.—In Devonshire and North Wales the stomach cancer soils do not differ significantly in average content from the controls, the mean levels being if anything below expectation. In Cheshire, however, the SC series shows an excess of 8·8 parts per million (t = 3·0). No appreciable differences appear for other cancer.

Lead.—Since the quantitative assessment of amounts of this element by the spectrographic method is difficult when the level exceeds 5 parts per million, the statistical comparison has been made by comparing proportions of the total soils having 5 or more p.p.m., and by the median values, these measures being unaffected by uncertainties as to the exact values in the upper part of the scale. The Devonshire data show a lower median for the stomach cancer series than for the controls, and 23 per cent of each series had 5 or more parts per million of lead. In the Cheshire-North Wales area there was no significant difference by either measure, but soils connected with non-gastric cancer show greater proportions with a high lead content than the controls, this series consisting mainly of intestinal cancers.

Copper.—The amounts of copper in the soils from the two Devonshire districts were determined by a colorimetric process in a separate acetic acid extract of the

soil, as described below. Table VI(b) shows that the mean content of the soils from houses where stomach cancer had occurred is higher by 0·50 p.p.m. than that of the controls. The ratios of cobalt to copper are about 0·18 in each group, and the ratios of nickel to copper are 0·7 in each group, giving no indication that copper has a counteracting effect. The ratio of zinc to copper, however, is 6·8 in the stomach cancer soils compared with 4·7 in the controls.

SUMMARY

Chemical and spectrographic study of garden soils in North Wales, Cheshire and two localities in Devonshire has established correlations between the amounts of certain constituents and the frequency of cancer of the stomach. Organic matter, zinc and cobalt are related positively and significantly with stomach cancer incidence but not with intestinal cancer, whilst chromium is connected with the incidence of each of these. The abnormal rates of stomach cancer in parts of North Wales are associated with long residence on soils whose organic content lies between definite limits. Soil rich in zinc or cobalt is found with excessive frequency where a case of stomach cancer has occurred but the geographical distribution of such soils appears to be unrelated to that of stomach cancer rates. Vanadium and iron show inconclusive relations with stomach cancer in one of the areas, whilst nickel, titanium and lead show no connection anywhere with this form of cancer.

METHODS OF TRACE ELEMENT ANALYSIS

Soils were examined for elements likely to be taken up by plants in micro- or trace quantity. The likelihood of this uptake is to some degree related not to the total quantity of each element in the soil, but rather to a combination of quantity weatherability and ease of solubility. To simulate this " availability for plants " it is customary to extract soils with very dilute acetic acid or neutral salt solutions or even with dilute solutions of ion complexing agents. For the present investigation N/2 acetic acid was chosen (20 g. soil/800 ml. acid) the acid being in contact with the soil with occasional shaking for 12 hours. Analysis of the acetic acid extract then followed closely the procedure of Mitchell (1945). This involves a separation of the micro-elements from those present in large amounts (e.g. K, Ca, Na, Mg) resulting in a concentrate of the micro-elements. The precipitate of all these elements is ultimately arced by direct current, the arc light being examined by a Hilger Large Quartz Spectrograph.

In the present investigation 30 mg. Al_2O_3, 2·5 mg. Fe_2O_3 and 0·4 mg. Cd. were introduced before precipitation to ensure consistent arcing and to provide elements for reference in the spectrogram. Cobalt, Nickel, Chromium, Vanadium, Titanium, Lead and Zinc were then determined quantitatively on the spectrogram using Iron, already found by chemical analysis, as a reference standard. Zinc was also determined by using the added Cadmium as reference, and it is this second value which has been used throughout the present work.

Available copper cannot be determined spectrographically because of limitations imposed by contamination. It was necessary therefore to devise a separate method, an adaptation of the colorimetric estimation by Zinc Dibenzyldithiocarbamate (Andrus, 1955). For this a fresh extract was prepared (20 g. soil by 800 ml. N/2 acetic acid). The extract was evaporated to dryness, oxidized with

a little nitric acid and dissolved in N. acetic acid. This was shaken with a solution of Zince Dibenzyldithiocarbamate in carbon tetrachloride and the intensity of colour produced in the latter by copper was estimated with a spectrophotometer.

We are greatly indebted to the Medical Officers of Health and their Assistants in Cheshire, the 5 counties of North Wales and the 2 districts of Devonshire for the collecting of soil samples and for supplying the information as to deaths from cancer and other causes. Our thanks are due also to the British Empire Cancer Campaign for supporting the work and to the Analysts and Technicians in the Department of Agricultural Chemistry at Bangor whose services have been provided by the Campaign.

REFERENCES

ANDRUS, S.—(1955) *Analyst*, **80**, 514.

DAVIES, R. I. AND WYNNE GRIFFITH, G.—(1954) *Brit. J. Cancer*, **8**, 56, 594.

HUXLEY, J.—(1958) ' Biological Aspects of Cancer ', London (Allen & Unwin).

MITCHELL, R. L.—(1945) ' Spectrographic Analysis of Soils, Plants and related Materials ', Technical Communication 44. Harpenden (Commonwealth Bureau of Soil Science).

STOCKS, P.—(1955) *Rep. Brit. Emp. Cancer Campgn*, **33**, 468.—(1956) *Ibid.*, **34**, 520.—(1958*a*) *Ibid.*, **36**, 342.—(1957) *Ibid.*, **35**, Supplement, p. 95. —(1958*b*) In ' Cancer ', London, (Butterworth), Chapter 4, Vol. 3, p. 153.

WALKLEY, A. AND BLACK, I. A.—(1934) *Soil Sci.*, **37**, 29.

SECONDARY TUMOURS OF THE HEART

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THE incidence of metastatic tumours in the myocardium was found to be 5 per cent in a series of 500 cancer necropsies reported by Willis (1952), who commented that the supposed infrequency of secondary growths in the heart wall was due to inadequate observation. Among the more recent papers on the subject are those of De Loach and Haynes (1953), Burnett and Shimkin (1954) and Goudie (1955).

The relative susceptibilities of different tissues to blood-borne metastases constitute one of the many interesting problems of cancer, and the recording of metastases in a collected series of cases can still serve a useful purpose.

The present study is based on the pathology of 50 cases of discrete secondary tumours of the heart. A few of these are comparatively recent, while the rest were found in the records of this hospital. Factors to be considered are the distribution of tumours within the heart, the types of associated primary tumours, and the incidence of associated metastases in other organs. In addition, one of the 50 cases will be presented in more detail, being of particular interest with regard to the "soil" hypothesis of tumour metastases (Willis, 1952).

The total incidence of secondary cardiac tumours cannot be given for this series, as the cases were not taken from a consecutive period over which the records are complete. Of the 50 cases, 28 were in males and 22 in females. Cases of leukaemia and of Hodgkin's disease have been excluded, as also have tumours directly invading the heart from adjacent structures. Metastases involving the endocardium and epicardium have been included, but those involving the parietal pericardium only have been excluded. The finding of neoplastic cells within lymphatic vessels of the heart has not been regarded as constituting true metastatic growth.

TABLE I.—*Distribution of Cardiac Metastases*

Part of heart involved	No. of cases
Right atrium	16
Right ventricle	24
Left atrium	9
Left ventricle	25
Interatrial septum	1
Interventricular septum	3
Epicardium	24
Epicardium only	7
Myocardium	43
Endocardium	9

DISTRIBUTION OF TUMOURS WITHIN THE HEART

The distribution of secondary tumours within the heart is shown in Table I. The metastases were solitary in 14 cases and multiple in 36 cases. The table shows a slight preponderance of tumours on the right side of the heart and the ventricles to be more frequently affected than the atria. The distribution of the growths reported by other writers (e.g. Yater, 1931; Scott and Garvin, 1939; De Loach and Haynes, 1953) has been variable, but Willis (1952) concluded that "all parts of the myocardium are equally prone to metastasis, and that the different parts are affected proportionately to their bulk". The table also shows a low incidence of septal involvement, but this is probably due to the septa not being specifically mentioned in reports on cases with multiple metastases.

TABLE II.—*Primary Tumours with Cardiac Metastases*

Primary tumour	No. of cases
Carcinoma	(33)
Bronchus	10
Skin	4
Kidney	4
Oesophagus	3
Stomach	2
Cervix uteri	2
Breast	1
Thyroid	1
Pancreas	1
Rectum	1
Vulva	1
Testis	1
Unknown origin	2
Malignant melanoma	5
Reticulosarcoma	4
Lymphosarcoma	1
Multiple myeloma	1
Fibrosarcoma (breast)	1
Osteogenic sarcoma (tibia)	1
Haemangio-endothelioma (liver)	1
Chorionepithelioma (uterus)	1
Teratoma (testis)	1
Neuroblastoma (adrenal)	1
Total	50

TYPES OF PRIMARY TUMOURS ASSOCIATED WITH CARDIAC METASTASES

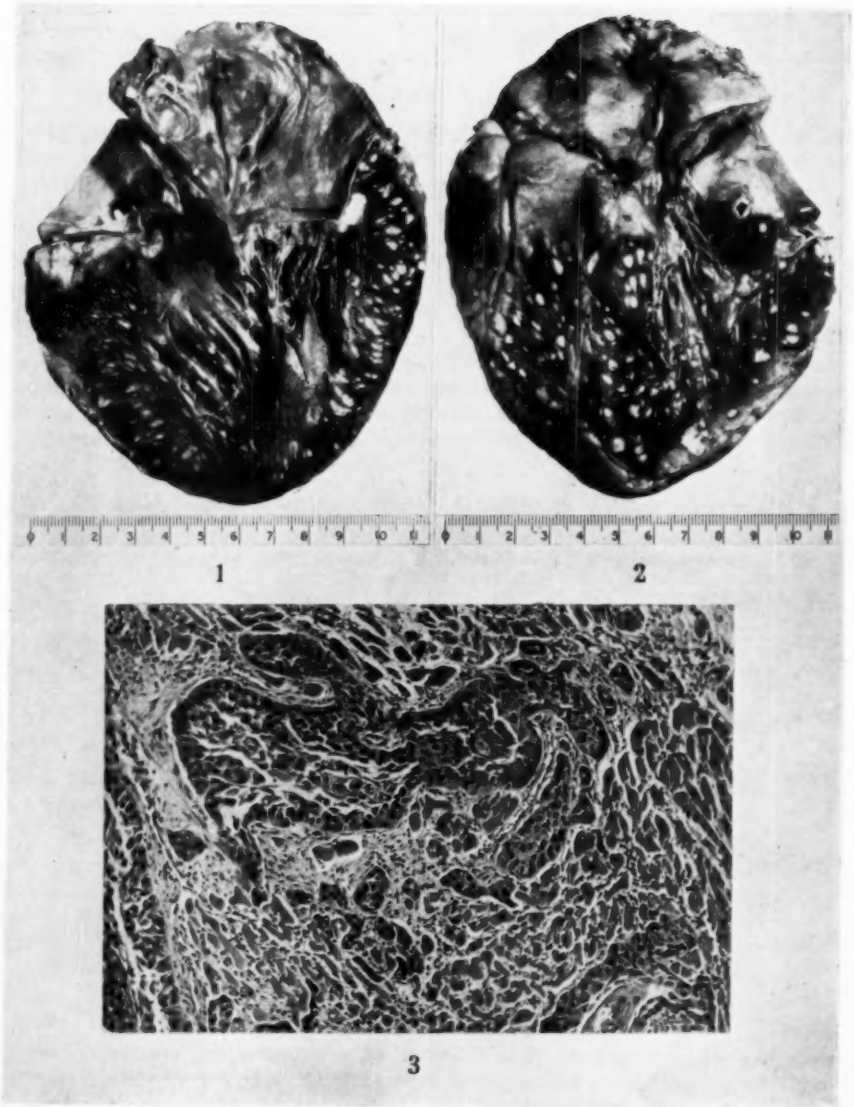
The various sites and types of the primary tumours which produced cardiac metastases are shown in Table II. The figures indicate no striking differences from those of other reported series (reviewed by De Loach and Haynes, 1953), the relatively high incidence of metastases from carcinoma of the bronchus and

EXPLANATION OF PLATE.

FIG. 1.—The heart sectioned to show multiple carcinomatous metastases in the left atrium and left ventricle. The arrow indicates calcification of the base of the posterior cusp of the mitral valve.

FIG. 2.—The heart sectioned to show metastases in the right ventricle as well as on the epicardial surface of the left ventricle.

FIG. 3.—Photomicrograph showing infiltration of left ventricular myocardium by squamous cell carcinoma. H. and E. × 85.



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from malignant melanoma and reticulosarcoma being typical. The proportion of cases of skin cancer is somewhat higher than usual, and there is a lower incidence of carcinoma of the breast. Of the 33 cases of carcinoma, 15 are squamous-celled, 11 are adenocarcinomas and seven undifferentiated.

METASTASES ASSOCIATED WITH SECONDARY TUMOURS OF THE HEART

In most of the reported cases of secondary cardiac tumours there have been widespread metastases in many other organs, although these have not usually been listed in detail, and in particular there has been a high incidence of associated malignant involvement of other intrathoracic structures (Lymburner, 1934; De Loach and Haynes, 1953). The tumours were also widely disseminated in the majority of cases in the present series, but in seven of the 50 cases there was no other apparent primary or secondary intrathoracic neoplastic involvement, and in two cases there was no definite information on this point.

The incidence of associated metastases in other organs is shown in Table III. Care was taken to exclude instances of direct neoplastic extension or lymphatic permeation as far as possible. For this reason secondary growths of the pleurae, peritoneum and skin have been excluded from the table, as also have lymph node metastases and serosal deposits on the abdominal organs.

TABLE III.—*Metastases associated with Secondary Tumours of the Heart*

Organ	No. of cases with metastatic involvement	Percentage of total cases (50)	Percentage of metastases in Willis' 500 Cancer necropsies
Liver	31	62	36
Kidneys	24	48	7·6
Lungs	23	46	20
Bones	19	38	13·6
Adrenals	18	36	9
Intestines	8	16	2
Spleen	8	16	3
Pancreas	7	14	3
Thyroid	6	12	4
Stomach	6	12	0·4
Brain	5	—	—
Ovaries	4	—	—
Oesophagus	3	—	—
Urinary bladder	3	—	—
Skeletal muscles	3	—	—
Gall-bladder	2	—	—
Meninges	2	—	—
Breast	1	—	—
Tongue	1	—	—
Tonsil	1	—	—
Testis	1	—	—
Subgluteal bursa	1	—	—

As metastatic tumours of the heart are usually accompanied by widespread metastases in other organs, the incidence of the latter should be higher than in unselected cases of malignant disease. The relatively high incidence of cardiac metastases from bronchogenic carcinoma would also tend to raise the frequency of secondary growths in such organs as the adrenals, kidneys and bones. In Table III are shown, for comparison, the percentages of metastases in certain organs

found by Willis (1952) in his 500 consecutive cancer necropsies. From this comparison it can be seen that in the present series there are particularly high percentages of associated metastases in the intestines, spleen, pancreas, thyroid and stomach. Of these organs the spleen is perhaps of most interest, as it was comparatively easy to check that the metastases were truly blood-borne and within the splenic substance. It is possible that some common factor exists to make such organs as the heart and spleen more susceptible "soils" for metastases in certain cases, these organs normally being relatively free of secondary tumours. Of the eight cases of associated metastases in the spleen there were four carcinomas, two melanomas, one fibrosarcoma and one haemangio-endothelioma.

In Lymburner's (1934) series of 52 cases of secondary cardiac tumours there were seven instances (13·5 per cent) of associated splenic metastases, a similarly high figure; six of these were carcinomas and one a sarcoma. Ritchie (1941) also reported one sarcomatous and two carcinomatous splenic metastases from 16 cases of metastatic tumours of the myocardium.

The following case illustrates an unusual distribution of secondary growths, with involvement of the heart and spleen.

CASE REPORT

H.H., a woman aged 63, had a radical vulvectomy for carcinoma of the vulva in January, 1957 at this hospital. Two right inguinal lymph nodes showed neoplastic infiltration, the tumour being a poorly differentiated squamous cell carcinoma. In April, 1958 the patient was admitted to another hospital with pyrexia, a skin rash and joint pains. Rheumatoid arthritis and erythema nodosum were diagnosed, and there was a good response to steroid therapy. Four months later, however, the patient became generally unwell and somewhat disorientated, and was re-admitted to this hospital on August 23rd. There was a past history of rheumatic fever at the age of 16 and alopecia totalis for 20 years. There had been eight pregnancies, including three miscarriages.

On examination there was no fever, but the patient was found to have auricular fibrillation, a slightly raised venous pressure, slight left ventricular hypertrophy, a systolic murmur indicating mitral incompetence, and Cheyne-Stokes respiration. The E.S.R. was 14 mm. in 1 hour (Westergren), and an electrocardiogram confirmed the auricular fibrillation and was reported to show left ventricular ischaemic changes. There was also a swollen right leg. Generalised carcinomatosis was suspected, and the patient died on the day after admission.

At autopsy there were metastases of squamous cell carcinoma in the abdominal lymph nodes, liver, spleen, peritoneum and heart, but none was found in any other intrathoracic structure or in the brain. Recent ante-mortem thrombi were present in the splenic and right femoral veins.

The heart weighed 470 g. and showed moderate left ventricular hypertrophy. Very numerous whitish metastases (measuring up to 0·8 cm. in diameter) were present in the epicardium, myocardium and endocardium of all four chambers, (Fig. 1 and 2) including the interatrial and interventricular septa. Part of the mitral ring and base of the posterior cusp of the mitral valve showed a zone of calcification measuring $1 \times 0{\cdot}5 \times 0{\cdot}3$ cm., which made the mitral orifice somewhat rigid. The remainder of the valve and the chordae tendineae showed no fibrous thickening, and the other valves were normal. The coronary arteries showed

slight atheroma but were not appreciably narrowed, and the aorta was only moderately atherosclerotic.

Microscopic examination shows extensive infiltration of the heart wall by moderately well differentiated squamous cell carcinoma (Fig. 3) with some keratinization and cell-nest formation. In the myocardium columns of tumour cells extend between the muscle fibres and are often closely related to the small blood vessels. There is a moderate degree of reactive fibrosis with associated chronic inflammatory cell infiltrations. No evidence of active rheumatism can be seen. The calcified area at the base of the mitral valve contains no carcinoma cells and is surrounded by dense fibrous tissue with a minimal inflammatory reaction.

The spleen weighed 245 g. and contained multiple metastases measuring up to 1 cm. in diameter. Microscopically, these are composed of fairly well differentiated squamous cell carcinoma with cell-nest formation. Several small arteries and veins contain recent ante-mortem thrombi.

The main interest in this case lies in the very extensive cardiac metastases in the absence of other intrathoracic metastases, together with the associated rheumatic history. Although the heart showed no evidence of active rheumatism or of extensive rheumatic scarring, the partial calcification of the mitral valve was probably rheumatic in origin. It is possible that the heart may have become more susceptible to metastasis on account of the previous rheumatism, but this interpretation can only be hypothetical, and the exact nature of such a susceptibility can only be conjectural at present.

A history of rheumatic fever was also found in two other cases of this series, in one of which there was scarring of the mitral valve. In two cases there was a history of scarlet fever. One of the cases reported by Scott and Garvin (1939) also had rheumatic heart disease.

SUMMARY

A study has been made of the pathology of 50 cases of discrete secondary tumours of the heart. Factors considered were the distribution of tumours within the heart, the types of associated primary tumours, and the incidence of associated metastases in other organs. One case with a rheumatic history, carcinoma of the vulva, very extensive cardiac metastases and no other intrathoracic neoplastic involvement, is described in more detail.

I wish to thank Professor J. W. S. Blacklock for helpful advice, Mr. J. W. Miller for histological sections, Mr. N. K. Harrison for the photographs, and Dr. G. S. Sansom for the photomicrograph.

REFERENCES

Burnett, R. C. and Shimkin, M. B.—(1954) *Arch. intern. Med.*, **93**, 205.
De Loach, J. F. and Haynes, J. W.—(1953) *Ibid.*, **91**, 224.
Goudie, R. B.—(1955) *Brit. Heart J.*, **17**, 183.
Lymburner, R. M.—(1934) *Canad. med. Ass. J.*, **30**, 368.
Ritchie, G.—(1941) *Amer. J. Path.*, **17**, 483.
Scott, R. W. and Garvin, C. F.—(1939) *Amer. Heart J.*, **17**, 431.
Willis, R. A.—(1952) ' The Spread of Tumours in the Human Body.' 2nd ed. London (Butterworth).
Yater, W. M.—(1931) *Arch. intern. Med.*, **48**, 627.

EFFECT OF THIO-TEPA ON ADVANCED MALIGNANT OVARIAN TUMOURS

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Treatment with Thio-TEPA has produced, in a significant number of patients suffering from very advanced carcinoma of ovary, marked temporary retrogression of growth and improvement of symptoms ; in a few cases temporary clinical disappearance of growth, ascites and disappearance of symptoms was achieved, and those patients resumed their normal activities enjoying life for the duration of control of growth. Our article contains observations made from January 1957 till June 1959, and an attempt of quantitative presentation of clinical results was made. Our observations confirm and emphasise previous reports.

Triethylene thiophosphoramide (Thio-TEPA) is an alkylating agent structurally related to nitrogen mustard and triethylene melamine and has a similar cytotoxic effect. The clinical effect appears to differ significantly.

Thio-TEPA was synthesised in the Lederle Laboratories.

Shay *et al.* (1953) reported marked improvement in some cases of chronic leukaemia and of Hodgkin's disease which were treated with Thio-TEPA ; they also reported an encouraging response in two patients suffering from metastases from mammary cancer.

These authors quoted and reviewed briefly the extensive experimental work of the Lederle group of workers and described their own work which led to the clinical trial.

Bateman published a detailed report on the clinical effect of Thio-TEPA in 99 cases of advanced malignant solid tumours (Bateman, 1955), and on 380 cases (Bateman, 1958).

Leonard, Israels and Wilkinson (1956) reported the effect of Thio-TEPA on Hodgkin's granuloma, chronic lymphatic leukaemia, polycythaemia and other reticuloses.

Many valuable reports have been published since 1953. A monograph of 1266 pages containing a series of papers on the chemistry, biological effects and clinical results of alkylating agents was published in April 1958 by the New York Academy of Science. This monograph also contains studies on the effect of Thio-TEPA on malignant disease and on haemopoiesis by Wright, Golomb and Gumport (1958), Ultman, Hayman and Gelhorn (1958), Olson (1958), Shay and Sun (1958), Bateman (1958), Alpert (1958), Moore (1958), Leone (1958) and other related papers with references to previous work and to other authors.

The effect of Thio-TEPA on Hodgkin's disease, chronic lymphatic leukaemia and other reticuloses has been reported to be similar, but not superior, to that of nitrogen mustard.

A few cases of various groups of solid malignant tumours have been treated with Thio-TEPA. Only in a very few of these has temporary effect of varying

degree and duration been reported. Further systematic observation and collection of data is necessary.

Reports have repeatedly been published on marked temporary regression of tumour and decrease of ascites, with improvement of symptoms, in advanced carcinoma of the ovary, confirming Bateman's original observations published first in 1955. Bateman also observed in a large proportion of cases of carcinoma of breast with secondaries, temporary regression of tumour masses, healing of ulceration, recalcification of bone lesions and control of effusions.

Recently Watson and Turner (1959) reported the favourable response of breast cancer to combined therapy with Thio-TEPA and testosterone propionate.

Thio-TEPA has a marked toxic effect on haemopoietic tissue which results in reduction of the number of leucocytes and of platelets; excessive doses will lead to irreversible changes of haemopoietic tissue, which may be fatal. Haematological control during treatment is therefore essential. In therapeutic doses the effect on haemopoiesis is reversible on cessation of treatment. Compared with nitrogen mustard the side effects, such as nausea and vomiting, are very slight and in most patients absent. Thio-TEPA has been injected intra-venously, intra-muscularly, into the tumour, into pleural or peritoneal cavity if fluid was present, and intra-arterially for regional treatment; it has also, though rarely, been given by mouth.

Bateman (1955) emphasized that Thio-TEPA was most effective when injected directly into the tumour.

We have treated 33 cases of malignant disease with Thio-TEPA:

Group A—carcinoma ovary—17 cases.

Group B—carcinoma breast—9 cases.

Group C—miscellaneous—8 cases (reticulum cell sarcoma 1, Brill Symmer's 1, carcinoma cervix 1, carcinoma bronchus 2, post cricoid carcinoma 1, myxosarcoma 1, secondary adenocarcinoma of groin of unknown primary +? secondaries in lungs).

Our observations on group B and C will be evaluated separately.

We present here the observations on the effect of Thio-TEPA in 17 cases of advanced carcinoma of ovary, which were treated in this department from January 1957 till June 1959. During those 29 months 63 cases were referred to the Radiotherapy Department and 52 cases were treated either with X-ray and radium or with X-ray only; most of the cases were referred for post-operative treatment.

Four of the 17 cases which were treated with Thio-TEPA were referred prior to January 1957.

All cases treated with Thio-TEPA were suffering from recurrence after surgery and radiotherapy, or the disease was too widespread for effective X-ray treatment and were inoperable.

The cases were not selected deliberately. All cases which became known to one of the authors (O. E.) who were suffering from advanced inoperable carcinoma of ovary and could not be treated with X-ray effectively were treated with Thio-TEPA; advanced but symptom-free cases were not treated.

It has been our aim to evaluate quantitatively the extent and duration of objective and subjective improvement.

For approximate quantitative appreciation of the clinical effect, we have expressed the degree of subjective and objective improvement in five grades and return to activity has been expressed separately, as shown in the key to the graphs.

Of the 17 cases treated 3 are alive and 14 died. Six showed no improvement (Table I), of those six, 3 were in a terminal stage at the start of treatment and had only two or one injection.

TABLE I.—*Cases in Whom Thio-TEPA had no Effect*

Case No.	Age	First symptom and date	Duration from first symptom to first treatment	Duration of observation from first treatment	Histology	Total dose and route	Degree of leukopenia during treatment	Remarks
12	41	October 1956. Enlarged abdomen and cough	4 months	24 weeks	Pleomorphic carcinoma	125 mg. into tumour	+	Died 24 weeks after first injection.
13	57	December 1957. Lassitude	10 months	2 weeks	Adeno-carcinoma	25 mg. intra-peritoneal	—	Died 2 weeks after first injection.
14	29	April 1957. Swelling of abdomen	7 months	2 days	Granulosa cell carcinoma	25 mg. intra-peritoneal	—	Died 2 days after first injection.
15	36	August 1957. Acute abdominal pain and vomiting	3 months	2 weeks	Arrhenoblastoma	50 mg. intra-peritoneal	—	Died within 2 weeks of injection.
16	20	July 1957. Abdominal pain	18 months	8 weeks	Anaplastic carcinoma ? arrhenoblastoma	60 mg. intra-venous	+	Died 8 weeks after first injection.
17	53	March 1958. Cough	3 months	12 weeks	Adeno-carcinoma	100 mg. intra-peritoneal	+++	Died 12 weeks after first injection.

Eleven cases showed a varying degree of improvement. Eight showed objective and subjective improvement. Three cases observed for only 11 to 12 weeks showed subjective improvement only (Table II).

The following case histories, with graphs, illustrate the clinical effect of Thio-TEPA.

CASE 1 (FIG. 1)

Age 55. Histology: Granulosa cell tumour. First symptom: February 1956, pain in left iliac fossa. July 1956: removal of bilateral carcinomatous tumours of ovary. The left ovarian tumour was incompletely removed because of extensive adhesion; secondary nodules on peritoneum and in omentum at the time of operation.

Post-operative X-ray treatment and one radium application to uterus was given in August/September 1956.

Prior to treatment with Thio-TEPA: severe abdominal pain; she could hardly keep any food down because of incessant vomiting. There was a large

tumour arising from the pelvis extending into abdomen to the level of the umbilicus.

31.1.57 treatment with Thio-TEPA commenced. She was given a course of 55 mg. in 3 injections into tumour over 3 weeks and later further injections as shown in graph.

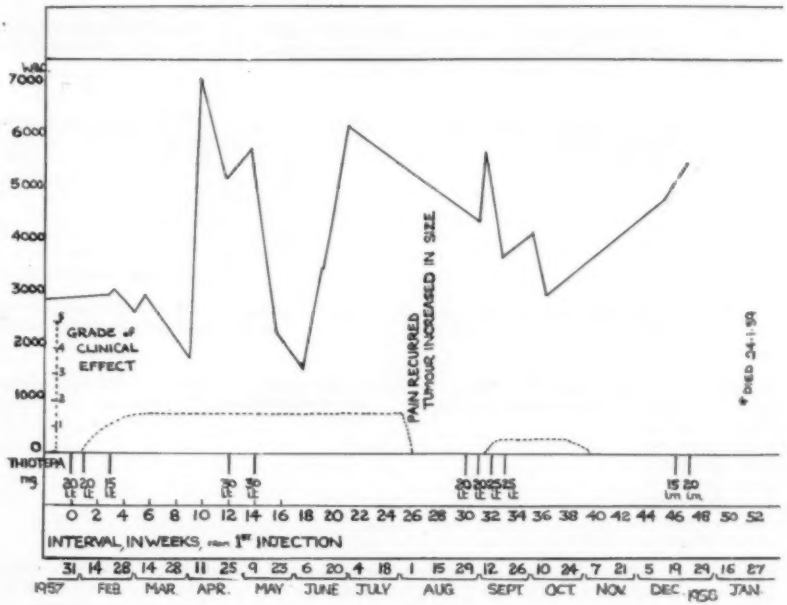
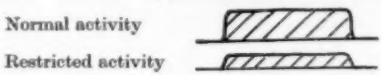


FIG. 1

Degree of clinical improvement - - - - - - - - - - - -

Grade 1. Improvement of symptoms.
2. Disappearance of symptoms.
3. Decrease in size of tumour or decrease of ascites.
4. Clinical disappearance of ascites.
5. Disappearance of tumour, of ascites and of symptoms.



Within one week from the start of treatment with Thio-TEPA improvement commenced, and in the third week vomiting had ceased, pain was markedly improved and the tumour had become smaller.

In the 9th week she was free from pain. The tumour was greatly reduced in size and could just be felt above the symphysis. She maintained improvement for 16 weeks, then the symptoms recurred and the tumour grew again rapidly in size.

A second course of 4 injections at weekly intervals to a total of 90 mg. was followed by improvement of pain and slight gain in strength for only 6 weeks,

TABLE II.—*Cases in Whom Thio-TEPA*

Case No.	Age	First symptom and date	Duration from 1st symptom to 1st treatment with Thio-TEPA in months	Histology	Time of observation from 1st treatment with Thio-TEPA in weeks	Total dose and route of administration
1	53	February 1956. Pain in left iliac fossa	11	Granulosa cell carcinoma	51	240 mg. into tumour and intramuscular
2	56	April 1956. Swelling of lower abdomen	29	Ditto	92	455 mg. intraperitoneal
3	44	March 1958. Pain in left abdomen	8	,,	47	375 mg. intraperitoneal and intramuscular
4	52	May 1958, Swelling of abdomen	6	Serous papillary adenocarcinoma	51	150 mg. intraperitoneal
5	55	December 1955. Malaise	37	Granulosa cell carcinoma	19	127 mg. intraperitoneal, intravenous and intramuscular
6	54	May 1956. Pain in right lower abdomen	33	Papillary cyst-adenocarcinoma	40	112·5 mg. intravenous
7	54	October 1958. Lassitude	4	Papillary adenocarcinoma	39	175 mg. intraperitoneal
8	52	December 1958. Swelling of abdomen	3	No histology	18	120 mg. intraperitoneal
9	43	November 1956. Pain in left pelvis	28	Granulosa cell carcinoma	30	100 mg. intramuscularly
10	55	December 1957. Vaginal bleeding	8	Poorly differentiated adenocarcinoma	16	215 mg. intraperitoneal and into tumour
11	57	June 1958. Swelling right groin	7	Papillary adenocarcinoma	12	200 mg. intraperitoneal

was Effective in Varying Degree

Clinical effect	Degree of leukopenia during treatment	Remarks June 1 1959	Further observations December 1959
Cessation of pain and vomiting and marked reduction in size of tumour for 15 weeks	+++	From 38th week onwards gradual deterioration. Died 51 weeks after first injection	..
Temporary disappearance of tumour ascites and symptoms—returned to normal activity. Improvement maintained for 32 weeks. Second course of injections for recurrence followed by disappearance of ascites, reduction of tumour and improvement of symptoms for 20 weeks. Third course of treatment started for recurrence, but no improvement observed yet	++	Still under observation.	Patient died 19.viii.59; 92 weeks after the first injection of Thio-TEPA.
Considerable regression of tumour. Strength, appetite improved. Greater sense of well-being	++	Alive and well doing light housework	Patient died 8.x.59; 47 weeks after the first injection of Thio-TEPA.
Marked regression in size of tumour. Ascites disappeared. All symptoms disappeared —returned to normal activity	++	Free from symptoms. Enjoying normal life.	Well until August 1959 then recurrence of growth and ascites. Further injections of Thio-TEPA followed by disappearance of symptoms for 8 weeks. Then rapid progress of disease and failing strength.
Disappearance of oedema of legs. Distension of veins of abdominal wall diminished. Improvement of nausea, vomiting and pain. Slight gain in appetite and weight	++	Developed recto-vaginal fistula in the 11th week. Under consideration for a further course when white count improves	Patient died 9.vi.59; 19 weeks after the first injection of Thio-TEPA.
Retrogression of pelvic tumour. Pain decreased. Appetite and strength improved	++++	Improvement maintained.	Improvement maintained. Two intramuscular injections of 30 mg. each were given in December.
Ascites decreased. Gained strength. Appetite improved. Sense of well-being	+	Doing her housework and working as part-time typist	Improvement maintained. Further 140 mg. in 6 intra-peritoneal injections given.
Appetite and strength improved	+	Symptoms improving	Patient died 20.vii.59; 18 weeks after the first injection of Thio-TEPA.
No measurable regression of tumour. Gained strength	++	Improvement of symptoms maintained to date	Patient died 4.xi.59; 30 weeks after the first injection of Thio-TEPA.
Pain and ascites diminished. Sense of well-being started within two weeks following first injection. Improvement maintained for 12 weeks. Slight reduction of tumour noted	++	Died 16 weeks after first injection of Thio-TEPA	..
Improvement in strength and appetite only for a short period	+++	Died 12 weeks after first injection of Thio-TEPA	..

then symptoms and signs recurred and she died in the 51st week after the first injection.

The effect on leucocytes was of particular interest. The number of leucocytes at the start of treatment was 3000, dropped to 1700 in the 9th week after the first injection of Thio-TEPA and quickly rose to 7100 when clinical improvement commenced.

Time of observation was 51 weeks.

CASE 2 (FIG. 2)

Age 56. Histology: well differentiated, probably granulosa cell carcinoma. First symptom: April 1956, swelling of lower abdomen.

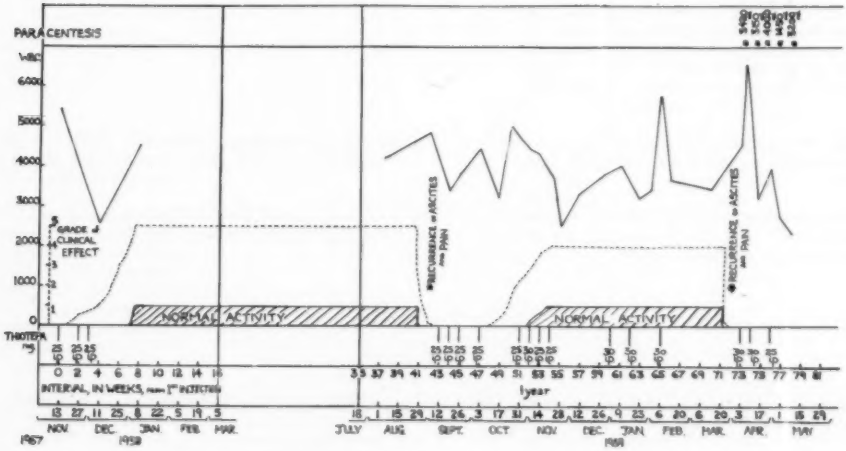


FIG. 2

Degree of clinical improvement - - - - - - - - - - - -

Grade 1. Improvement of symptoms.
2. Disappearance of symptoms.
3. Decrease in size of tumour or decrease of ascites.
4. Clinical disappearance of ascites.
5. Disappearance of tumour, of ascites and of symptoms.

Normal activity

Restricted activity

13.8.56 subtotal hysterectomy and bilateral oophorectomy for bilateral ovarian carcinoma. Secondary nodules on pelvic peritoneum and peritoneum overlying the bladder. 13.8.56 2 pints of blood transfused.

September/October 1956 post-operative X-ray treatment and one radium application to uterus.

Prior to Thio-TEPA: pelvic pain, abdominal distension and bleeding from the umbilicus on slight trauma. Examination after removal of 3000 ml. of ascites by paracentesis on 12.11.57: nodular mass in pouch of Douglas extending into both sides of pelvis. Secondary tumour, 2 cm. diameter, in umbilicus.

Commencing on 13th November 1957, she was given the first course of Thio-TEPA, 3 intra-peritoneal injections of 25 mg. each, in 3 weeks. She vomited occasionally during the first 7 days; from the second week onwards she gained strength, her appetite improved and she felt well. From the 8th week onwards there was no evidence of disease and she was free from symptoms. Definite decrease in size of the tumour was observed in the 7th week after the first injection and in the 11th week all growth and ascites had disappeared. She led a normal life doing all her housework for approximately 8 months, then pain, ascites, tumour of pelvis and umbilicus reappeared rapidly.

She was given further injections of Thio-TEPA commencing with a course of 4 injections of 25 mg. each in 4 weeks, and later further injections as shown in Fig. 2.

Six weeks after the start of the second course she again gained in strength, attained a sense of well-being and her appetite improved; her symptoms disappeared completely and she returned to her normal activities for $3\frac{1}{2}$ months. During this period ascites disappeared, the growth had decreased in size but did not disappear; then pain and ascites recurred rapidly and frequent paracentesis became necessary. Further injections were given as shown in the graph.

The patient died 19.8.59, 92 weeks after the first injection of Thio-TEPA.

CASE 3 (FIG. 3)

Age 44. Histology: granulosa cell carcinoma producing pseudoadenomatous appearances.

First symptoms: March 1958, pain in left abdomen.

Operation: 24.9.58: gross ascites, abdomen filled with bilateral multilocular ovarian cystic tumours with a large amount of solid and friable tissue. Metastases on pelvic peritoneum and uterus. Both ovarian new growths removed.

Prior to Thio-TEPA: 27.10.58 ascites; firm mass of growth in upper third of rectovaginal septum and pouch of Douglas; large mass in left pelvis and a large mass in left lumbar region extending into left hypochondrium.

Treatment with Thio-TEPA commenced on 10.11.58: a course of 84 mg. was given in 5 injections in 10 days, and a second course of 116 mg. commenced 8 weeks after the first injection.

From the 10th day onwards she gained strength, her appetite improved and later on she felt well and was doing light housework except when ascites had accumulated. From the 14th week onwards tumour decreased in size but did not disappear. In the 19th week a paracentesis of abdomen was necessary, subsequently she was well and doing light housework until the 25th week when ascites and symptoms recurred. Further injections were given as shown in Fig. 3.

The patient died 8.10.59, 47 weeks after the first injection of Thio-TEPA.

CASE 4 (FIG. 4)

Age 52. Histology: serous papillary adenocarcinoma of ovary.

First symptom: May 1958, swelling of abdomen.

Operation 13.8.58 : 4500 ml. of ascitic fluid removed. Right ovarian cystic tumour of 12 cm. diameter removed. Peritoneal metastases present.

13.8.58 : 4 pints of blood transfused.

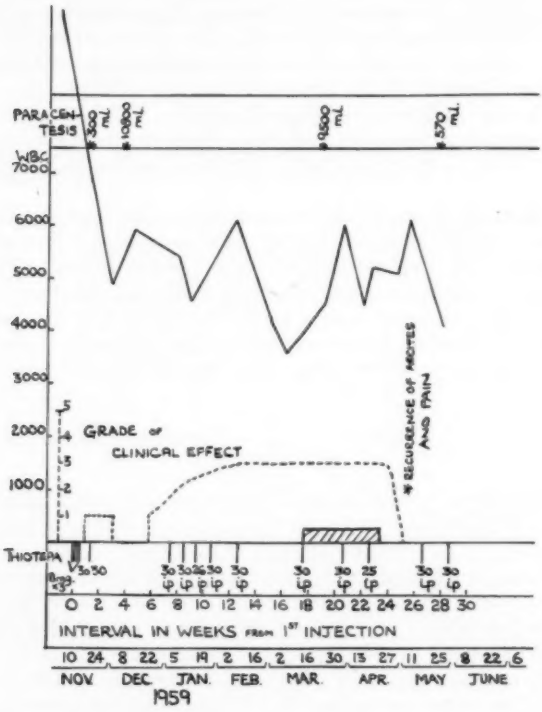


FIG. 3

Degree of clinical improvement - - - - - - - - - - - -

Grade 1. Improvement of symptoms.
2. Disappearance of symptoms.
3. Decrease in size of tumour or decrease of ascites.
4. Clinical disappearance of ascites.
5. Disappearance of tumour, of ascites and of symptoms.

Normal activity

Restricted activity

Post-operative X-ray treatment September/October 1958, could not be completed because of poor general condition.

Prior to Thio-TEPA : November 1958, examination under anaesthesia after removal of 5700 ml. of ascites : a mass consisting of matted spherical tumours filling pelvis and lower abdomen extending into upper abdomen on the right side.

Main symptoms : lassitude and gnawing abdominal pain.

On 22.11.58 the first course of Thio-TEPA commenced ; 100 mg. were given in 4 intra-peritoneal injections of 25 mg. each at weekly intervals, and she was given a further 25 mg. in the 16th and 17th week after the first injection.

In the 6th week decrease in size of tumour mass was observed, and in the 10th week the tumours had disappeared except for one 4 cm. diameter lump at the

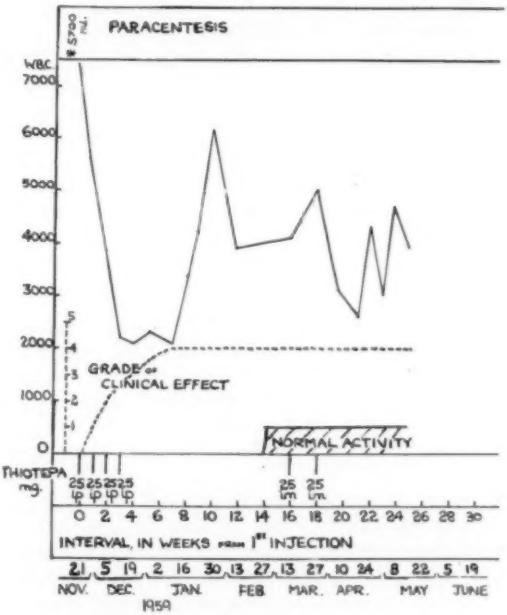


FIG. 4

Degree of clinical improvement - - - - - - - - - - -
Grade 1. Improvement of symptoms.
2. Disappearance of symptoms.
3. Decrease in size of tumour or decrease of ascites.
4. Clinical disappearance of ascites.
5. Disappearance of tumour, of ascites and of symptoms.

Normal activity

Restricted activity

level of umbilicus which was still palpable. At that time ascites had disappeared and did not re-accumulate during the time of observation. The lump at the level of umbilicus continued to decrease in size but did not completely disappear. From the second week onwards her appetite improved, she gained strength and attained a sense of well-being, and from the 18th week onwards she could do all her housework, and was enjoying a normal life.

She remained well and vigorous and the tumours decreased further in size until August 1959. In August the tumours, ascites and symptoms reappeared.

A further 3 intra-peritoneal injections of 30 mg. each were given followed by improvement of symptoms and return to her activity for 8 weeks. Since then the growth increased in size, she lost strength and flesh rapidly.

CASE 5 (FIG. 5)

Age 55. Histology : granulosa cell carcinoma.
First symptom : December 1955, malaise.
13.1.56 : removal of a large left multilocular cystic ovarian growth.

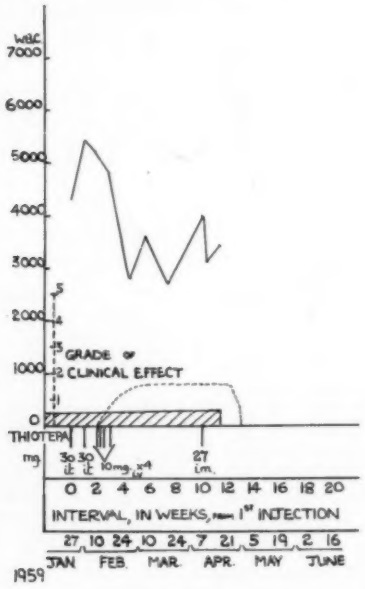


FIG. 5

Degree of clinical improvement - - - - - - - - - - - -
Grade 1. Improvement of symptoms.
2. Disappearance of symptoms.
3. Decrease in size of tumour or decrease of ascites.
4. Clinical disappearance of ascites.
5. Disappearance of tumour, of ascites and of symptoms.

Normal activity
Restricted activity

May 1957 : large fixed hard nodular mass of growth in pouch of Douglas and left pelvis. X-ray treatment and one radium application to uterus was followed by only slight regression of growth and temporary alleviation of pain lasting approximately 6 months.

Prior to treatment with Thio-TEPA : January 1959, extensive growth involving rectum and rectovaginal wall causing stenosis of rectum, oedema of legs,

vulva and buttocks and gross distension of veins of abdomen. She had nausea, occasional vomiting, anorexia, frequent watery stools and sometimes loss of control, frequency of micturition; abdominal pain and tiredness even at rest: she was still doing a little light housework.

Treatment with Thio-TEPA started on 27.1.59. She was given a course of 6 injections to a total of 100 mg. in 3 weeks and then one 27 mg. intra-muscularly 9 weeks after the first injection.

From the second week onwards pain and nausea decreased, appetite improved and she gained a little in strength.

The tumour decreased in size slightly and the oedema of legs, vulva, buttocks and the distension of abdominal veins disappeared. A rectovaginal fistual developed in the 12th week. The distension of abdominal veins and oedema of thighs and pain reappeared in the 14th week.

A colostomy was done and one pint of blood was transfused on 4.6.59.

The patient died 9.6.59; 19 weeks after the first injection of Thio-TEPA.

CASE 6 (FIG. 6)

Age 54. Histology: papilliferous cystadenocarcinoma.

First symptom: May 1956, pain in right lower abdomen.

15.9.58: complete removal of left ovarian growth, incomplete removal of large friable growth of right ovary which was adherent to uterus and pelvic wall.

17.9.58: 2 pints of blood transfused.

October/December 1958: one radium application to uterus and X-ray treatment to pelvis.

Prior to treatment with Thio-TEPA: a large mass in the pelvis involving uterus pressing on rectum. Constant pain in abdomen and in right shoulder for which she required alternatively papaveretum gr. $\frac{1}{3}$ or pethidine 100 mg. 4 hourly day and night. She was very ill and confined to bed.

5.12.58; 2 pints of blood transfused.

Treatment with Thio-TEPA commenced on 10.2.59.

She was given at first 62·5 mg. in 7 intra-venous injections in 17 days.

From the 6th week onwards her appetite improved, she gained strength and pain decreased. Decrease in size of the tumour commenced in the 8th week; in the 9th week abdominal pain had almost completely disappeared. She was discharged home and in the 12th week she could walk around and help a little in her household.

A further 2 intra-muscular injections of 30 mg. of Thio-TEPA were given in December. Objective and subjective improvement was maintained.

CASE 7 (FIG. 7)

Age 54. Histology: papillary carcinoma of ovary.

First symptom: October 1958, lassitude.

12.11.58 operations: gross ascites. Omentum and peritoneum studded with nodules, pelvis filled with growth. During the 2 months preceeding the first injection of Thio-TEPA she needed paracentesis on 6 occasions at intervals of 5 to 21 days.

Prior to Thio-TEPA : gross distension of abdomen due to large masses of growth and ascites ; loss of flesh ; lassitude, vomiting, swelling of legs when abdomen distended by ascites.

On 12.2.59 treatment with Thio-TEPA commenced. She was given as the first course 150 mg. divided into 5 intra-peritoneal injections at weekly intervals. Further injections as shown in Fig. 7.

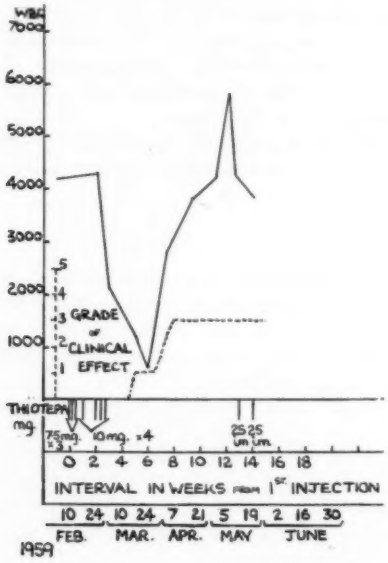
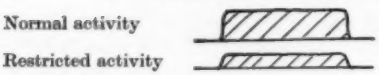


FIG. 6

Degree of clinical improvement - - - - - - - - - - -

Grade 1. Improvement of symptoms.
2. Disappearance of symptoms.
3. Decrease in size of tumour or decrease of ascites.
4. Clinical disappearance of ascites.
5. Disappearance of tumour, of ascites and of symptoms.



During the first 2 weeks after the first injection a paracentesis was done on two occasions. From the 3rd week onwards ascites decreased slightly. She gained strength, her appetite improved and she felt well.

She is doing her shopping and working part time as a typist.

Change in the size of the tumour is difficult to assess because of ascites.

The time of observation since the first injection of Thio-TEPA was 15 weeks on June 1st 1959.

From June 1st till October 9th 1959 she was given a further 140 mg. in 6 intra-peritoneal injections. Paracentesis abdominis was necessary once in October 1959.

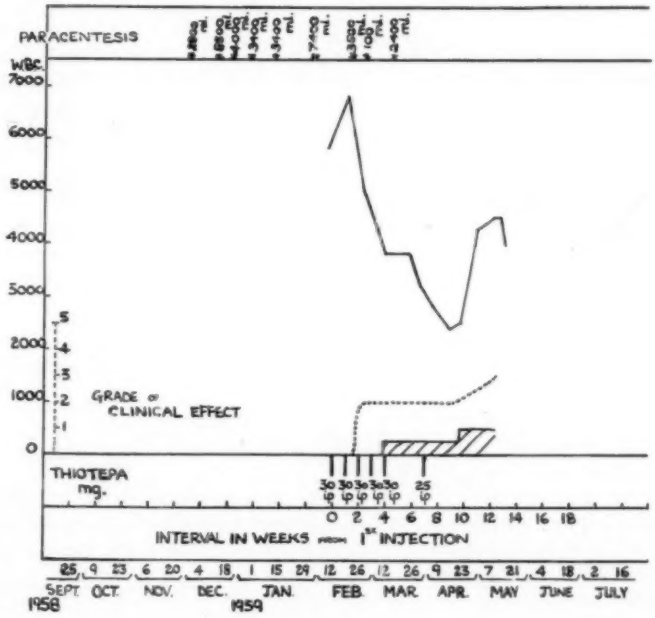


FIG. 7

Degree of clinical improvement - - - - - - - - - - - -

Grade 1. Improvement of symptoms.
2. Disappearance of symptoms.
3. Decrease in size of tumour or decrease of ascites.
4. Clinical disappearance of ascites.
5. Disappearance of tumour, of ascites and of symptoms.

Normal activity

Restricted activity

At the end of December ascites and abdominal tumour were present; she felt quite well except at the time when a paracentesis had become necessary; she continues doing part time work as a typist and also doing light housework.

CASE 8

Age 55. No histology.

First symptom: December 1958, swelling of abdomen.

Prior to treatment with Thio-TEPA: distension of abdomen, shortness of

breath, loss of weight and anorexia. A paracentesis of chest and abdomen was done 5 times during the preceding 2 months.

Examination after removal of 7 litre of fluid by paracentesis of abdomen : large nodular growth in pelvis filling pouch of Douglas extending upwards to the level of umbilicus. Multiple large lobulated tumour masses in abdomen. Liver enlarged.

Treatment with Thio-TEPA commenced on 18.3.59. She was given 4 intra-peritoneal injections at weekly intervals to a total of 120 mg. Ascites was removed by paracentesis on 4 occasions during the 6 weeks following the first injection. From the 5th week onwards she gained strength and her appetite improved.

In the 9th week ascites and pleural effusion increased causing dyspnoea and therefore fluid was removed by paracentesis of chest.

The patient died on 20.7.59, 18 weeks after the first injection of Thio-TEPA.

CASE 9

Age 45. Histology : granulosa cell carcinoma.

First symptom : November 1956, pain in left pelvis.

March 1957 : hysterectomy and bilateral salpingo-oophorectomy for carcinoma of ovary.

April/May 1957 : post-operative X-ray treatment to pelvis.

July 1958, secondary node in operation scar treated with X-ray.

Mass of growth in pelvis treated with X-ray November/December 1958.

Prior to treatment with Thio-TEPA : a fixed hard mass in left pelvis extending almost to the level of umbilicus. 4 cm. diameter subcutaneous metastatic tumour in abdominal wall in upper end of operation scar. Liver enlarged. Slight pain in right iliac fossa, slight backache and nausea.

Treatment with Thio-TEPA commenced on 18.3.59. She was given at first 3 injections intra-muscularly of 25 mg. each at weekly intervals.

The leucocytes dropped from 6400 to 2300 in the 3rd week ; a further 25 mg. was given after the leucocytes had risen to 4200 at the end of the 6th week.

From the second week onwards she gained strength and attained a sense of well-being. No definite change in the size of the tumour has been observed.

The patient died 4.11.59, 30 weeks after the first injection of Thio-TEPA.

METHOD

The crystalline Thio-TEPA was dissolved in normal saline 10 mg. per ml. and stored no longer than 14 days in refrigeration. The route and dose in this series varied. If ascites was present the injections were given into the peritoneal cavity. A small quantity of 2 per cent procaine was injected locally before giving Thio-TEPA intra-muscularly or into the tumour. The dose varied from case to case depending on the patients strength, the state of nutrition, haematological status and on the route. From our experience we came to the conclusion that 65 to 120 mg. given in fractionated doses over three to four weeks is an effective therapeutic dose. Intravenously Thio-TEPA was given at the rate of 7 to 10 mg. per day to a weekly maximum dose of 30 mg. Injections of Thio-TEPA are discontinued when the graph of total leucocytes showed a downward trend following previous injections.

Principles of maintenance treatment are yet to be developed. We intend to give a second course in all cases which show a good response, at the latest 3 to 5 months after the first course.

It is of great value that most cases can be treated as out patients.

EFFECT AND REACTIONS

In 4 of our cases of advanced carcinoma of the ovary, treatment with Thio-TEPA was followed by striking temporary regression or clinical disappearance of the growth, reduction or disappearance of ascites and marked improvement of symptoms with return to activity and enjoyment of life. Three patients had definite marked temporary regression of growth and improvement of symptoms only. The maximum duration of improvement after one course was 8 months. Second and subsequent courses were less effective than the first course.

Improvement in strength, appetite and sense of well-being, soon after the start of treatment, was noted in all cases in whom Thio-TEPA had any clinical effect.

Though symptoms may improve during or shortly after the second week, decrease in size of tumour and of ascites occurs mostly after 6 to 8 weeks.

All our cases who showed a marked improvement had temporary leukopenia following treatment.

Decrease in the number of granulocytes and platelets commenced in the second or third week, and recovered quickly to normal. Petechiae and cutaneous haemorrhages were observed in two cases at the time of maximum depression of haemopoiesis and subsided spontaneously.

The effect of Thio-TEPA on leucocytes was remarkable in case 1 in whom the number of leucocytes at the start of treatment was 3000, dropped to 1700 8 weeks after treatment and rose to 7100 one week later. The fall and later the rise of the number of leucocytes coincided with retrogression of growth and improvement of symptoms.

Only one case had side reactions such as nausea and vomiting. There were no unpleasant local or general reactions in any of the other cases.

DISCUSSION

Thio-TEPA has a marked growth inhibiting effect in some cases of carcinoma of the ovary. The effect is only temporary. Thio-TEPA does not supersede surgery or radiotherapy in the treatment of ovarian cancer. It is, however, of definite value in some cases in whom all other treatments have failed. In such cases Thio-TEPA may still achieve temporary control of disease to such an extent that the patient may enjoy a normal life and may return to normal activity for many months. The duration of temporary control will, one can reasonably expect, increase with greater experience and by the various means of protecting the haemopoietic tissue.

Small repeated transfusions of blood given daily for a few days before, and during treatment with Thio-TEPA, may reduce the proliferative and mitotic activity of haemopoietic tissue to a resting phase. In the resting phase the bone marrow may be less sensitive to a radio-mimetic and antimitotic agent.

If this concept is correct, 50 ml. of blood given daily should reduce the bone marrow activity. A plan for investigation in this direction is in preparation.

From what has already been done in this field, and from our own experience, we conclude that ovarian carcinoma occupies the foremost position in the spectrum of activity of Thio-TEPA over the whole range of malignant disease.

In many cases of advanced cancer classification by histology is often difficult and sometimes debatable. It is likely that some cases which appear to be primary malignant tumours may, in fact, be secondaries from a growth elsewhere. If this is true the selective effect of Thio-TEPA on primary ovarian carcinoma is probably higher than it appears from our series. It is of immediate interest to correlate sensitivity of the various groups of ovarian cancer to chemotherapeutic agents with their natural history and histology, and to study each group according to their sensitivity.

A plan of study of the effectiveness of chemotherapy as an adjuvant to surgical treatment of cancer has been described by Shimkin and Moore (1958).

Two investigations are being carried out using Thio-TEPA in resectable carcinoma of stomach and nitrogen mustard in resectable cancer of lung. One of the aims of the plan is to ascertain whether chemotherapeutic agents are more effective when the tumours are small and clinically not obviously established.

The discovery of the effect of Thio-TEPA on carcinoma of ovary has opened new and promising paths in the field of experimental and clinical research on ovarian carcinoma.

SUMMARY

The effect of Thio-TEPA in 17 cases of advanced carcinoma has been described.

Objective improvement has been achieved in 8 cases, some of whom have returned to their normal activity for several months. The clinical effect is temporary. In all cases the effect was associated with transient leukopenia.

We are most grateful to the Lederle Laboratories, Division of Cyanamid of Great Britain Limited, for their generous supply of Thio-TEPA and for literature and references.

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REFERENCES

ALPERT, L. K.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 1072.
BATEMAN, J. C.—(1958) *Ibid.*, **68**, 1057.—(1955) *New Engl. J. Med.*, **252**, 879.
LEONARD, B. J., ISRAELS, M. C. G. AND WILKINSON, J.—(1956) *Lancet*, ii, 1017.
LEONE, L. A.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 1081.
MOORE, G. E.—(1958) *Ibid.*, **68**, 1074.
OLSON, K. B.—(1958) *Ibid.*, **68**, 1018.
SHAY, H., LARAFONETIS, C., SMITH, N., WELDOW, I. and SUN, C. H.—(1953) *Arch. intern. Med.*, **92**, 628.
Idem AND SUN, C. H.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 1046.
SHIMKIN, M. B. AND MOORE, G. E.—(1958) *J. Amer. med. Ass.*, **167**, 1710.
ULTMAN, J. E., HAYMAN, G. A. AND GELLHORN, A.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 1007.
WATSON, G. W. AND TURNER, R. L.—(1959) *Brit. med. J.*, i, 1315.
WRIGHT, J. C., GOLOMB, F. M. AND GUMPORT, S. L.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 937.

THE ISOLATION OF EPITHELIAL CELLS FROM NORMAL AND NEOPLASTIC COLON

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THE comparison of the enzyme activities of normal and neoplastic tissue is usually unsatisfactory because it is carried out on blocks of tissue containing a mixed population of cells. The ratio of the epithelial to the stromal component varies not only as between normal and neoplastic tissue, but also within different normal and neoplastic tissues. Attempts have been made to resolve this difficulty by counting the relative numbers of each kind of cell (Chalkley, 1943 ; Rosenthal and Drabkin, 1944 ; Sibley and Fleisher, 1955). However, this is a tedious procedure and it does not overcome the problem of determining the amount of enzyme activity in each different component of the tissue.

Clearly, the epithelial component of both the tumour and the homologous normal tissue should be isolated in order to compare their enzyme activities. In this paper such a procedure is described for the isolation of the epithelial cells of the mucosa and of carcinomata of the colon.

METHODS AND RESULTS

Preparation of tissue

The colon is obtained within 30 minutes of excision. A longitudinal incision is made along its whole length, the mucosa is wiped free from faecal matter with the aid of filter paper and the subperitoneal fat is removed. The specimen is then pinned out on a board and the mucosa is separated from the submucosa at or above the level of the muscularis mucosae by means of a stiff paint or tooth brush the bristles of which have been cut to 0·5 cm. in length. The sheets of mucosa obtained in this way are transferred to ice-cold 0·25 M sucrose.

The everted edge of the tumour is removed above the levels of the adjacent normal mucosa externally and the ulcer base internally. The tissue so obtained is compressed between sheets of filter-paper in order to remove mucus and necrotic debris and it is then trimmed to remove the connective tissue and any areas of haemorrhage.

The pieces of normal mucosa and tumour are finally washed three times in ice-cold 0·25 M sucrose. At this stage the histological appearance is that seen in Fig. 1, 2 and 3. In Fig. 1 a section of the mucosa superficial to the muscularis mucosae is seen, and in Fig. 2 there is a section of the growing edge of a carcinoma of the colon. In both of these sections the epithelial cells and stroma are clearly defined from each other. In Fig. 3 there is a surface view of a piece of fresh mucosa mounted under a coverslip in 0·25 M sucrose. The acini and the individual cells of which they are comprised are seen in plan *in situ* in the lamina propria. When the

microscope is focussed up and down on such a preparation the lumina of the acini have the appearance of tunnels.

Isolation of epithelial cells

The epithelial cells are now isolated by a combination of two processes, namely (1) Disruption of the tissue, and

(2) Repeated differential centrifugation of the resultant suspension. The details are set out below.

All materials and equipment are kept at 0° C. and all procedures except weighing are carried out at this temperature. A histological examination is carried out as follows on each sample of the suspension removed for high-speed centrifugation. The sample is removed from the centrifuge tube by means of a Pasteur pipette which is filled from the surface of the suspension. This ensures that the deeper layers, i.e., the last to be removed, are situated in the distal part of the pipette. At stages 1(c), 2(b) and the corresponding stage under 3 of the scheme below, one drop from the tip of the pipette is placed on a glass slide, stained and examined for the presence of stroma. If any stroma is seen a suitable volume of the suspension is returned to the centrifuge tube, and again the drop in the tip of the pipette is examined. This procedure is repeated until all of the stroma is eliminated. The remainder of the contents of the pipette is then discharged into tube C for high-speed centrifugation. After a little experience only 3–4 histological examinations are needed.

Scheme for the isolation of epithelial cells from stroma

1. (a) 1 g. tissue plus 9·0 ml. 0·25 M sucrose are placed in the tube of a Potter-Elvehjem type homogeniser (tube A), and the pestle* is forced down and up 20 times.

(b) The suspension is centrifuged (in tube A) at 2500 r.p.m. for 3 minutes and the supernatant liquid plus the upper half of the "fluffy" layer (see later) are removed to a second tube, B.

(c) Tube B is centrifuged at 2500 r.p.m. for 3 minutes and the upper three quarters of the suspension are removed to tube C.

(d) Tube C is centrifuged at 5000 r.p.m. for 5 minutes, and the clear supernatant liquid is transfered to tube B, mixed with the contents remaining as under (c) and poured into the homogeniser.

2. (a) The pestle is forced down and up a further 80 times and the contents of the homogeniser are returned to tube B.

(b) Tube B is centrifuged at 2500 r.p.m. for 3 minutes and the upper half of the suspension is transferred to tube C.

(c) Tube C is centrifuged at 5000 r.p.m. for 5 minutes and the clear supernatant liquid is transferred to tube B which is swirled in order to mix the contents.

3. The procedures 2(b) and (c) are repeated until the number of free nuclei remaining in the deposit in tube B is negligible, that is, approximately 5 per high-power field. Usually it is necessary to repeat procedures 2(b) and (c) twice more.

* A plastic pestle is used. The clearance between the wall of the tube and the pestle should be such that when the pestle is held vertically with the tube in position and containing water, the tube slowly falls.

Disruption of the tissue

The movement of the pestle against the homogeniser tube compresses the tissue in such a way that the epithelial cells are expressed from, and/or stripped off, the stromal tissue. The appearance in the fresh state of the suspension so produced from the mucosa is seen in Fig. 4, 5 and 6. In Fig. 4 the expression of the acini from the lamina propria is shown at an early stage and in Fig. 5 an acinus is shown lying free surrounded by the nuclei of disrupted cells. The appearance of the lamina propria is seen in Fig. 6 in which the complete removal of the acini is demonstrated. A section of the tumour in the same stage of preparation appears in Fig. 7 in which clumps of tumour cells and fragments of connective tissue are visible.

Differential centrifugation

As a result of centrifugation the suspension is resolved into the following layers from below upwards: pieces of connective tissue containing a few acini, clumps of cells, single cells, nuclei, mitochondria and microsomes. The connective tissue, cells and nuclei are usually held together as a pink fluffy layer by the mucus which is released from the goblet cells. This makes it difficult to separate the various components of the suspension. However, separation is achieved, as outlined above, by a system of centrifugations followed by washing of the deposit with the top layers of the supernatant liquid. The end-result is apparently a complete resolution of most of the epithelial cells from the stroma.

The appearance of sections of the epithelial cells and nuclei prepared in this way from the mucosa are seen in Fig. 8 and 9. Many of the acini and separate cells appear to be intact and even where the cell membrane was broken the nuclei do not seem to have been damaged. Most of the cells and nuclei are clearly epithelial, but there is a small percentage of nuclei whose origin it is impossible to ascertain. It is unlikely that many of these nuclei arise from the connective tissue because, as shown in Fig. 10, the lamina propria does not appear to have been disturbed by the procedure used to express the acini.

The same general comments apply to the sections prepared from the epithelial cells and connective tissue residue of the carcinoma. In Fig. 11 both the malignant cells and the free nuclei appear to be intact. In Fig. 12 the connective tissue of the tumour is still cellular but owing to the lack of regular architecture it is difficult to be certain that none of the cells from the connective tissue was shorn off in the homogeniser.

DISCUSSION

This appears to be the first occasion on which epithelial cells have been isolated from the supporting elements in human tissue on a large scale. Hele (1953) prepared epithelial cells from the small intestine of the rat using a similar technique.

There are at least three criticisms of the method. First, the epithelial cells may be contaminated with connective tissue cells. As already pointed out this does not seem to be a likely or important source of error. Second, some of the contents of the cells of the stroma may leak into the sucrose medium. At present there is no way of determining whether this has happened, but it is probably minimal at 0° C. Third, all of the epithelial cells are not recovered. Complete recovery was

not attempted because it is believed that the less the disruption of the tissue commensurate with an adequate yield of epithelial cells, the less is the likelihood of the release of cells from the connective tissue and of leakage of enzymes from these cells.

There appears to be no reason why the method should not be applied to other tissues, for example, to small intestine in man and to tumours in which the epithelial cells are readily removed from the stroma in man or animals. Several attempts were made to obtain a preparation of epithelial cells in this way from human gastric mucosa; they failed because in the stomach the acini do not separate readily from the lamina propria.

SUMMARY

A method for the isolation of the epithelial cells from human colonic mucosa and carcinoma is described.

My thanks are due to Dr. Basil Morson and the staff of the Research Department at St. Mark's Hospital and to surgical colleagues at several hospitals for the supply of tissue, to Dr. I. Doniach for his advice and criticism and to Mr. W. Brackenbury for the photomicrographs.

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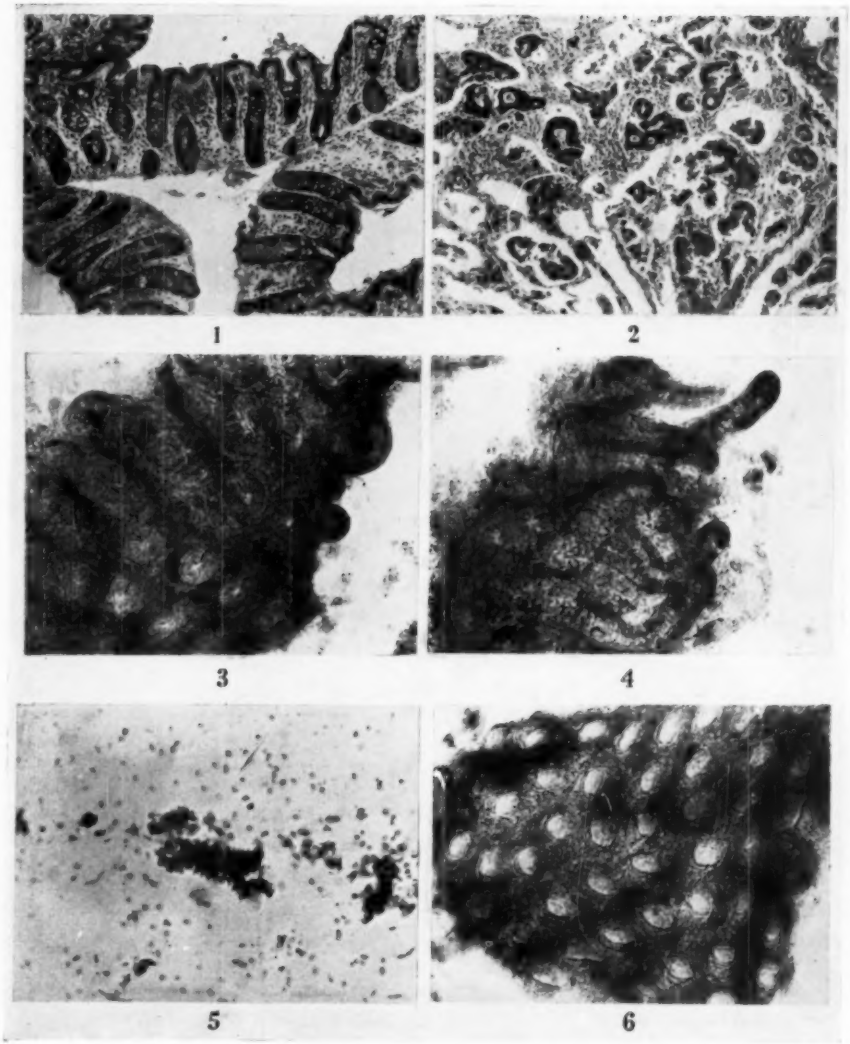
REFERENCES

CHALKLEY, H. W.—(1943) *J. nat. Cancer Inst.*, **4**, 47.
HELE, M. P.—(1953) *Biochem. J.* **55**, 857.
ROSENTHAL, O. AND DRABKIN, D. L.—(1944) *Cancer Res.*, **4**, 487.
SIBLEY, J. A. AND FLEISHER, G. A.—(1955) *Ibid.*, **15**, 609.

EXPLANATION OF PLATES

FIG. 1.—Section of the mucosa of the colon prepared from the sheets of mucosa stripped off the submucosa with the aid of a stiff brush. × 60.
FIG. 2.—Section of the growing edge of a carcinoma of the colon showing the tissue from which the epithelial cells of the carcinoma are isolated. × 60.
FIG. 3.—Surface view of the mucosa showing the acini in plan (unfixed). × 57.
FIG. 4.—Expression of the acini from the lamina propria at an early stage (unfixed). × 57.
FIG. 5.—Extruded acinus lying among the intact nuclei of epithelial cells (unfixed). × 80.
FIG. 6.—Surface view of the lamina propria from which the acini were expressed (unfixed). × 57.
FIG. 7.—Clumps of tumour cells and connective tissue as seen during disruption of a carcinoma (unfixed). × 60.
FIG. 8.—Section of the epithelial cells and nuclei isolated from the lamina propria. Note that many of the acini are intact. × 60.
FIG. 9.—High-power view of Fig. 8 showing that the cells are apparently intact. × 600.
FIG. 10.—Section of the lamina propria showing that the connective tissue cells are still *in situ*. × 58.
FIG. 11.—Section of some epithelial cells of a carcinoma isolated from the stroma. × 180.
FIG. 12.—Stroma of a carcinoma after isolation of the epithelial cells showing that the connective tissue cells are still *in situ*. × 60.



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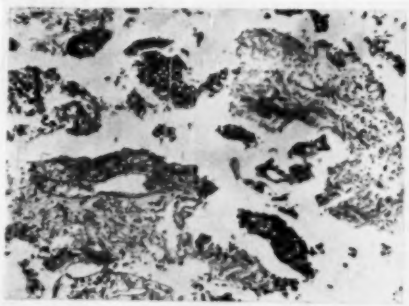

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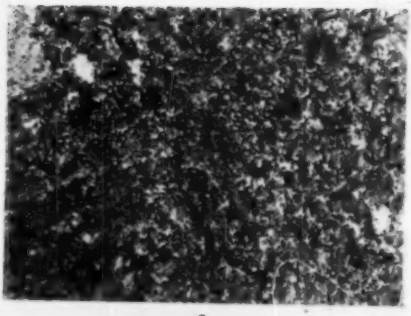

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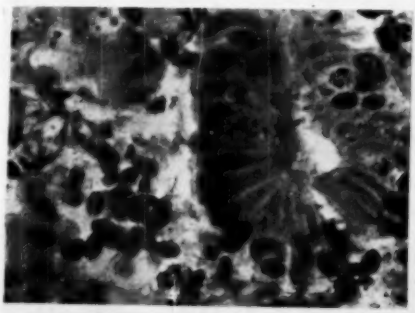

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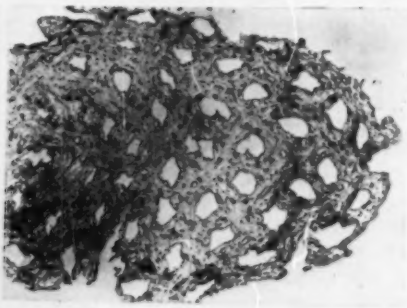

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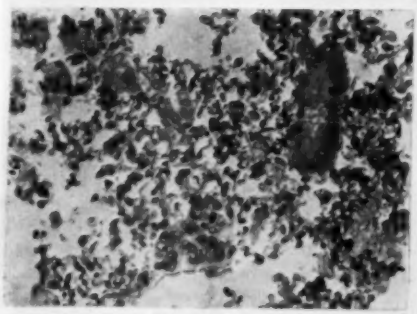

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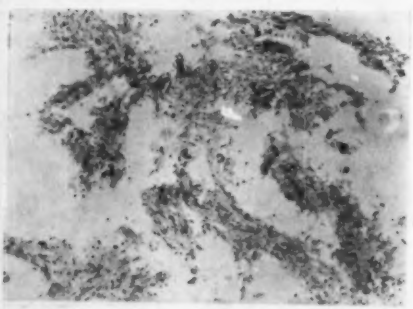

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BASOPHIL ADENOMATA IN THE RAT HYPOPHYSIS AFTER GONADECTOMY

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THE production of neoplasia by prolonged stimulation of endocrine tissues by physiological mechanisms is not only of great intrinsic interest but has some clinical significance because of the probability that such mechanisms are operative in the production of neoplasms in man. An analogy has been drawn by Purves (1956) between the production of basophil adenomata in the rat hypophysis by conditions that cause hyalinisation of normal basophil cells, and the association of basophil adenomata with Crooke's cell changes in the human hypophysis.

The basophil cells of the pars anterior of the rat hypophysis are composed of two functionally distinct groups. One group secretes thyrotrophin and is strongly and specifically stimulated by thyroxine deficiency. The other group secretes gonadotrophins and is strongly and specifically stimulated by gonadal hormone deficiency. The two groups have been termed thyrotrophs and gonadotrophs respectively (Purves and Griesbach, 1951). Thyrotrophs and gonadotrophs can be differentiated by their responses to staining by certain dyes. After staining by aldehyde-fuchsin the thyrotrophs appear as *beta* cells with positively stained granules, while the gonadotrophs appear as *delta* cells since their granules are not stainable by aldehyde-fuchsin (Halmi, 1950). There is evidence that the gonadotrophic group is heterogeneous and comprises two specific types—FSH and LH cells—which secrete follicle stimulating and luteinising hormone respectively (Purves and Griesbach, 1954, 1955).

Chronic stimulation of the thyrotrophs of the rat pars anterior by thyroxine deficiency induced by goitrogen administration gives rise to basophil adenomata. These adenomata are beta cell adenomata composed of angular cells resembling normal thyrotrophs and containing granules which are stainable by aldehyde fuchsin. Evidence for the view that such adenomata are derived from thyrotrophs has been summarised by Bielschowsky (1955). It is notable that the cells of the adenomata do not show any of the hyalinisation changes that affect the majority of the thyrotrophs in the surrounding pars anterior tissue in rats receiving goitrogen (Purves, 1956).

Basophil adenomata appearing in the hypophyses of rats after gonadectomy have been reported by Houssay, Houssay, Cardeza and Pinto (1955) and by Griesbach and Purves (1956). These adenomata were composed of cells which resembled normal gonadotrophs but did not show the hyalinisation which affects the normal gonadotroph after gonadectomy. Specific granulation was often present in amounts conferring strong staining properties on the adenomata. The granulation was of the delta type since, like that of normal gonadotrophs,

it was not stained by aldehyde-fuchsin (Griesbach and Purves, 1956; Purves, 1956). Houssay *et al.* (1955) considered that oestrogen or androgen secreted by adrenalcortical tumours was involved in the production of the hypophysial adenomata which appeared in their gonadectomised rats. This view was in accordance with the mechanism proposed by Woolley and Dickie (1949) for the production of hypophysial adenomata in castrated mice. We, at one time, considered that gonadectomy at an early age might be necessary for the production of basophil adenomata since we had not observed such adenomata in an earlier study of the long term effects on the hypophysis of rats of gonadectomy performed on sexually mature rats. Neither of these hypotheses is sustained by the observations reported in this paper.

MATERIALS AND METHODS

Both male and female rats of our Wistar strain were gonadectomised in 1953 and 1954 in connection with another investigation. The long term survivors were examined for the presence of hypophysial tumours. Some of these rats had been gonadectomised at 6 weeks of age, (Group A), some at 3 months, (Group B), and some at 9 months, (Group C).

In addition to the above animals, an experiment was begun in 1954 to determine the effect of exogenous oestrogen on the response of the hypophysis to gonadectomy. This experiment included :

Group D. 24 rats (12 male, 12 female) gonadectomised at 6 weeks of age and simultaneously implanted with 10 mg. cholesterol pellets containing 2·5 per cent stilboestrol.

Group E. 24 rats (12 male, 12 female) treated similarly to Group D, but with cholesterol pellets containing 0·5 per cent of stilboestrol.

Group F. 30 females implanted at 6 weeks of age with 10 mg. cholesterol pellets containing 2·5 per cent stilboestrol.

Group G. 26 female rats implanted at 6 weeks of age with 10 mg. cholesterol pellets containing 0·5 per cent stilboestrol.

In 1956 an experiment was begun to determine the relation of the adrenal cortex to hypophysial adenoma formation. This experiment comprised :

Group H. 60 rats (30 male, 30 female) gonadectomised at 6 weeks of age.

Group I. 60 rats (30 male, 30 female) gonadectomised and adrenalectomised at 6–7 weeks of age.

Group J. 38 female rats adrenalectomised at 6–7 weeks of age.

The adrenalectomised animals were given 1 per cent saline as drinking-water.

In some groups, one half of the animals were killed at 18 or 19 months after gonadectomy and the remainder at 24 months. In Groups D, E, F, G, some animals were killed at earlier ages to determine the time of appearance of tumours.

Animals which appeared to be unhealthy or obviously sick were killed and examined grossly. Their hypophyses were not examined microscopically. The healthy animals were killed with coal gas and the hypophyses removed and fixed in sublimate-formaline (9 : 1). The adrenals were taken for histological examination.

The histology of the hypophyses

The hypophyses were imbedded in paraffin and sectioned, beginning from the inferior surface and continuing until the pars intermedia and pars nervosa were reached. The sections were distributed among four microscope slides, of which one was stained by Crossmon's (1937) modification of the Mallory stain, one by Gomori's (1950) aldehyde-fuchsin, and two by the McManus periodic acid-Schiff (PAS) method as modified by Purves and Griesbach (1951*a*). In the latter investigations we have used instead of Gomori's (1950) formula, a stable aldehyde-fuchsin powder* prepared according to Gabe's (1953) method. This powder (0·5 g.) was dissolved in 100 ml. 70 per cent alcohol, filtered and acidified with 2 ml. of concentrated hydrochloric acid. This solution may be used immediately after preparation and keeps for 4–6 weeks at room temperature.

Before staining, the sections were oxidised with Lugol's iodine solution for 20 minutes. Permanganate oxidation, as used by Gabe (1953), destroys the specificity of the staining of the basophil cells of the rat pars anterior.

In general the sections were cut at 5 μ thickness, but a certain number were always taken at 2·5 μ thickness. These thin sections were used to obtain photomicrographs showing satisfactory detail. For visual examination the 5 μ. sections were generally preferable because of the stronger staining.

All the sections were searched systematically on the mechanical stage of a binocular microscope and the number and type of tumours found in each hypophysis recorded.

Tumours were recognisable as areas composed of a single cell type with signs of displacement and compression of the surrounding tissue. Evidence of cellular abnormality was present in all the nodules classified as tumours.

Uniformly large or uniformly small cell size, excessive variability of cell size, excessive variability of nuclear size and chromatin content and the presence of abnormal mitotic figures were considered to indicate cellular abnormality. The absence of hyalinisation in basophil tumours in gonadectomised animals in which normal gonadotrophs were hyalinised was also considered an indication of abnormality.

Tumours composed of cells containing glycoprotein granules as revealed by PAS staining were classified as basophil tumours. These were subdivided on the basis of the response to aldehyde-fuchsin staining. Tumours whose granules were stained by aldehyde-fuchsin were classified as beta cell tumours and those whose granules were not stained by aldehyde-fuchsin were classified as delta cell tumours. Tumours composed of cells which contained acidophil granules (stained red by the Crossmon stain) and those whose cells contained no recognisable specific granulation were grouped together in the acidophil-chromophobe class.

RESULTS

In general, survival of the gonadectomised rats was good and most of the animals were in a good state of health when killed.

The survival of adrenalectomised rats was, however, not good despite the supply of saline as drinking water. Of the 60 rats which were adrenalectomised in addition to gonadectomy only 22 survived for the length of time necessary

*" Aldehyde-fuchsin " prepared by Farbenfabriken Bayer, Leverkusen, and presented to us by Dr. H. Harms to whom we express our thanks.

for comparison with the non-adrenalectomised controls. The fatality rate was greatest at the age of 4–8 weeks when many of the rats died either suddenly or more slowly with oedema of the legs and fluid in the abdominal and thoracic cavities. The kidneys frequently showed pathological changes. In nearly all the rats of this group who had lived for 18–24 months after operation, adrenal cortical tissue without the medulla was found. In only three animals was no adrenal tissue seen by gross inspection of the region of the anterior pole of the kidney. It seems to be certain that the animals survived because adrenal cortical tissue had been provided either by small remnants not removed during operation or by accessory adrenal tissue growth. The adrenal tissue found post mortem never exceeded the size of a normal adrenal nor did it contain tumour tissue microscopically.

Tumour incidence

The results are summarised in Table I. The results of both sexes have been combined since in none of the groups was there any significant difference in the incidence of tumours in the sexes. There was no significant difference between Group D receiving 0·25 mg. stilboestrol and Group E receiving 0·05 mg. stilboestrol. Therefore the results of these two groups have been combined.

The main finding was a high incidence of delta cell tumours in all gonadectomised rats that survived more than 15 months. This incidence was not markedly affected by the age at which gonadectomy was performed, or by the other treatments (stilboestrol implantation, adrenalectomy) which were combined with gonadectomy in Groups D, E and I. This type of tumour is quite uncommon

TABLE I.—*Adenomata in the Hypophysis of Gonadectomised Rats*

Group of rats	Age of rats (months)		Additional treatment	Number of rats		Adenomata found		Analysis according to cell type		
	At start	At death		Total	With Adenomas	Total	Per hypophysis	Delta	Beta	Acidophil Chromophobe
Gonadectomised										
A	1½	19½–25½	—	29	28	98	3·4	91	0	7
B	3	22–35	—	21	21	89	4·2	78	0	11
C	9	28–36	—	8	8	39	4·9	34	0	5
D & E	1½	14½–19½	Stilboestrol 0·25 mg. 0·05 mg.	24	21	46	1·9	44	0	2
H	1½	19½–25½	—	38	36	40	2·2	68	0	15
I	1½	19½–25½	Adrenalectomy	22	18	83	1·8	29	3	8
Total gonadectomised				142	132	395	av. 2·8	344	3	48
Not gonadectomised										
F	1½	18½	Stilboestrol 0·25 mg.	24	14	14	1	2	0	12
G_1	1½	11½	0·05 mg.	15	0	0	—	0	0	0
G_2	1½	17½	0·05 mg.	7	4	4	1	0	0	4
J	1½	15½–19½	Adrenalectomy	15	5	5	1	1	1	3
K	—	18–24	—	68	15	19	1·3	5	6	8
K	—	16½–17½	—	21	4	4	1	0	0	4
Total non-gonadectomised (excluding G_1)				135	42	46	av. 1·1	8	7	31

in non-gonadectomised rats. In 135 animals in Groups F, G, J and K only 8 possible delta cell tumours were seen and, as explained later, of these, 3 tumours contained little glycoprotein and were not easily classified.

Beta cell tumours were seen in 3 gonadectomised animals and 7 non-gonadectomised animals. Gonadectomy, therefore, did not appear to produce or inhibit the formation of beta cell tumours.

Acidophil-chromophobe tumours were found in 48 animals of all groups examined at ages over 15 months. Gonadectomy did not have any marked effect on the incidence of this type of tumour.

Multiple tumours were frequently found. In general, the number of tumours found per hypophysis increased with age.

Varieties of delta cell tumour

Altogether 344 delta cell tumours were seen in gonadectomised rats. After studying these tumours we found that they could be allocated to one or other of three fairly well-defined groups. There were 61 tumours in 49 animals in which the cells, by the coarsely-flocculent nature of their granulation, closely resembled normal peripheral gonadotrophs (FSH cells). These tumours we will call " peripheral gonadotroph-like tumours " (Fig. 1, 3, 7, 9, 11, 13).

More prevalent were tumours composed of cells closely resembling central gonadotrophs. Altogether 241 " central gonadotroph-like tumours " were found in 117 animals. In tumours of this group a more or less uniform dispersion of the basophil granules resulted in a diffuse pink coloration of the cytoplasm. In addition there might also be some coarse PAS positive granules adjacent to the Golgi body but the appearance of the peripheral cytoplasm was characteristic. As in normal central gonadotrophs the diffuse PAS reaction was often stronger in the interior of the Golgi region and the negative image of the Golgi was, in consequence, especially conspicuous (Fig. 2, 4, 5, 8, 10, 14).

Forty-two delta cell tumours were composed predominantly of lightly granulated cells. In these cells the PAS reaction was usually confined to the interior of the Golgi body, the rest of the cytoplasm being quite pale. Occurring among cells of this appearance were always some cells with diffuse pink coloration throughout the cytoplasm (Fig. 6, 12, 15, 16). Three delta cell tumours found in non-gonadectomised animals were of this lightly-granulated type.

Distribution in the anterior lobe

The different types of tumour were not distributed at random within the anterior lobe but showed specific preferences for certain situations. The peripheral gonadotroph-like tumours were confined to the inferior surface and the periphery of the pars anterior, especially at the anterior border where the portal vessels enter from the stalk. This distribution corresponds to the distribution of the peripheral type of gonadotroph. In contrast the central gonadotroph-like tumours were most often found in the anterior lobe at a point where the portal vessels have broken up into normally-sized capillaries. Central gonadotroph-like tumours were not, however, confined to this region and were found, though in lower numbers, in other regions with the exception of the posterior edge. The beta cell type of tumour was rarely seen in this investigation. When it was found it was situated in the central area (Fig. 17) or posterior to the

triangle formed by the pars intermedia. The acidophil-chromophobe cell tumours had a preference for the posterior edge of the anterior lobe. They were never seen in the area where the large portal vessels enter the anterior lobe.

The structure of the adenomata

In most adenomata a well-defined tissue structure was developed. This usually took the form of a grouping of the cells into small rounded masses enclosed by basement membranes as in the normal pars anterior tissue. In some tumours this arrangement was more regular and well-defined than in the normal rat pars anterior in which the arrangement is somewhat irregular. In other tumours a well-differentiated structure was present which differed from the normal structure of the pars anterior. In these tumours cords and sheets of cells were present, separated from each other by irregular blood-filled spaces lined by endothelium (Fig. 5, 14, 18). An absence of any formed structure (anaplasia) was found most commonly in the acidophil-chromophobe group of tumours and was associated with the more extreme degrees of cellular abnormality which were common in this group.

Abnormality of the blood vessels was common in both basophil and acidophil-chromophobe tumours. Greatly dilated vessels and large blood-filled spaces of irregular shape were often seen in both groups. Only in the acidophil-chromophobe group were haemorrhagic tumours seen, but in this group almost all tumours showed haemorrhagic areas or signs of old haemorrhage in the form of macrophages containing altered blood pigment. It is noteworthy that the accumulations of pigment in the macrophages give an intense coloration with the PAS reaction. These cells are easily distinguished from basophil cells by the brown colour of the pigment.

Evidence of secretion by acidophil-chromophobe tumours

In 20 of the 48 gonadectomised animals with acidophil-chromophobe tumours there was evidence of lactogenic hormone secretion. The mammary glands showed extensive development apparent to naked-eye examination, and evidence of milk secretion was seen. In some there were milk-filled cysts and dilated ducts from which milk spurted when the glands were cut. In others histological examination showed dilated ducts filled with eosinophilic secretion. These effects were found in 10 males and 10 females ; the mammary changes appeared the same in both sexes. Secreting mammary tissue was not found in association with any of the acidophil-chromophobe tumours in the non-gonadectomised groups in this series.

Adrenal changes in gonadectomised rats

In all gonadectomised rats an unusual degree of hyperaemia was found, especially in the reticular zone of the adrenal cortex. The blood vessels in this region were widely dilated, forming large blood-filled spaces (Fig. 19, 20). A few small adenomata were seen in the cortex but none resembling the large tumours described by Houssay *et al.* (1955).

There was no evidence of secretion of oestrogen or androgen from the adrenal cortex or tumours arising therefrom in any of our gonadectomised animals. The seminal vesicles and prostates of all the males were atrophic, and the uteri of the

females were small and unstimulated. Only in some females implanted with stilboestrol pellets and killed one to two months later were enlarged fluid-filled uteri found, an effect which is ascribed to the exogenous oestrogen.

Phaeochromocytomas were quite frequent in the adrenal medullae of the rats examined but are not considered to be relevant to this investigation.

DISCUSSION

The significant finding in these experiments is a high incidence of basophil tumours with the staining properties of delta cells in all groups of gonadectomised rats which survived in good condition for 15 or more months. The production of these tumours must be ascribed to the continuous maximal stimulation of gonadotrophs by gonadal hormone deficiency. We have already referred to the fact that two types of gonadotroph can be distinguished—a peripherally-situated type which we believe secretes FSH and a centrally-situated type which we believe secretes LH. It is noteworthy that the majority of delta cells tumours in gonadectomised rats could be classed as resembling in cytological appearance one or other of the two normally occurring gonadotrophs. Also the tumours of the two types were differently distributed in the anterior lobe, the distribution of each type being similar to the distribution of the normal gonadotrophic cell which it most resembled and from which it was presumably derived.

In the bat (*Myotis myotis*) Herlant (1956) found that distinctive FSH and LH secreting cells could be easily differentiated by staining reactions. Unfortunately, in the rat the properties of the granules of the two types of gonadotroph are so similar that we have been unable to distinguish between them by staining reations, although a solubility difference in the granulation has been shown by Barrnett, Ladman and MacAllaster (1955). We have not, therefore, been able to show that the two varieties of delta cell tumour which we have seen contain granules of a different quality.

The lightly-granulated delta cell tumours warrant some discussion. From the fact that cells of this appearance can be found in tumours predominantly composed of central gonadotroph-like cells and that occasional well-granulated cells can be found in tumours predominantly formed of lightly-granulated cells we are satisfied that these tumours are basophil tumours. It should be noted that the tendency to retain glycoprotein granules within the Golgi body is a characteristic of normal central gonadotrophs under degranulating conditions. It is our opinion that most of the lightly-granulated delta cell tumours, in which the PAS reaction was confined mainly to the interior of the Golgi body, were central gonadotroph-like tumours in which differentiation was more marked than in the easily recognisable central gonadotroph-like tumours. The tendency for PAS positive material to be confined to the Golgi region was associated with a greater degree of pleomorphism of the cells, and in many cases it appeared that an area composed of cells of this form had developed in an adenoma of the typical central gonadotroph-like type. Such appearances are interpreted by us as an indication that lightly-granulated tumours are often derived from typical central gonadotroph-like tumours by an additional neoplastic change.

Woolley and Dickie (1949), as well as Houssay *et al.* (1955) have stressed the fact that in their gonadectomised animals hypophysial adenomata were found only in animals which had neoplastic changes in the adrenal cortex and they

EXPLANATION OF PLATES

FIG. 1.—Low power view of a basophil adenoma in the pars anterior of a rat killed 27 months after gonadectomy. The adenoma is situated at the periphery of the pars anterior and the cells resemble normal peripheral gonadotrophs. Note also the dilated blood vessels. PAS × 60.

FIG. 2.—Low power view of a basophil adenoma occupying a central position in the pars anterior of a rat killed 19½ months after gonadectomy. The cells of the adenoma contain varying amounts of granulation. The tissue structure of the adenoma is similar to that of normal pars anterior tissue. The cells resemble normal central gonadotrophs. PAS × 60.

FIG. 3.—Low power view of the pars anterior of a rat killed 32 months after gonadectomy. On the right is a well-granulated basophil adenoma whose cells resemble peripheral gonadotrophs. Slightly to the left of centre is a basophil adenoma of moderate granule content, composed of cells resembling central gonadotrophs. PAS × 60.

FIG. 4.—Low power view of a basophil adenoma in the pars anterior of a rat killed 27 months after gonadectomy. Cells of moderate granule content, resembling central gonadotrophs, form the left portion of the adenoma. The right side of the adenoma is composed of cells of low granule content, the PAS reaction being confined to the Golgi region. PAS × 60.

FIG. 5.—Low power view of an adenoma in the central region of the pars anterior of a rat killed 19 months after gonadectomy. The tissue structure is abnormal, being composed of strands of cells separated by blood-filled spaces which are white in the photo-micrograph. The cells of this adenoma resemble central gonadotrophs. PAS × 60.

FIG. 6.—Low power view of an adenoma in the central region of the pars anterior of a rat killed 24 months after gonadectomy. The cells of this adenoma are basophils of low-granule content, the PAS reaction being, in the main, confined to the Golgi region of the cell. Tissue structure is abnormal, consisting of well-defined cell cords separated by blood-filled clefts (white in the photomicrograph). PAS × 60.

FIG. 7.—High power view of peripheral region of the pars anterior of a rat killed 3 months after castration showing the characteristic features of peripheral gonadotrophs affected by castration changes. Note the coarse, strongly PAS positive basophil granulation. A large proportion of the cells are hyalinised forming typical "signet-ring" cells. The negative image of the Golgi body is visible in some of the cells as a white ring or crescent. PAS × 400.

FIG. 8.—High power view of the central region of the pars anterior of a rat killed 3 months after castration. As compared with Fig. 7 the central gonadotrophs are seen to have a more uniformly distributed, finer granulation, giving the cytoplasm a pink colour in the PAS stained section and appearing grey in the photomicrograph. The negative image of the Golgi body is visible in most of the cells. In the hyalinised cells the granulation still retains its diffuse appearance. PAS × 400.

FIG. 9.—High power view of the cells of a basophil adenoma in the periphery of the pars anterior of a rat killed 24 months after gonadectomy. The cells have a resemblance to unhyalinised peripheral gonadotrophs. PAS × 400.

FIG. 10.—High power view of the cells of a centrally-situated basophil adenoma in the pars anterior of a rat killed 29 months after gonadectomy. The cells resemble unhyalinised central gonadotrophs. Note the prominent negative images of the Golgi bodies and the even distribution of fine granulation through the cytoplasm. Tissue structure is normal. PAS × 400.

FIG. 11.—High power view of part of the adenoma shown in Fig. 1. The cells resemble unhyalinised peripheral gonadotrophs, with a reduced granule content. PAS × 400.

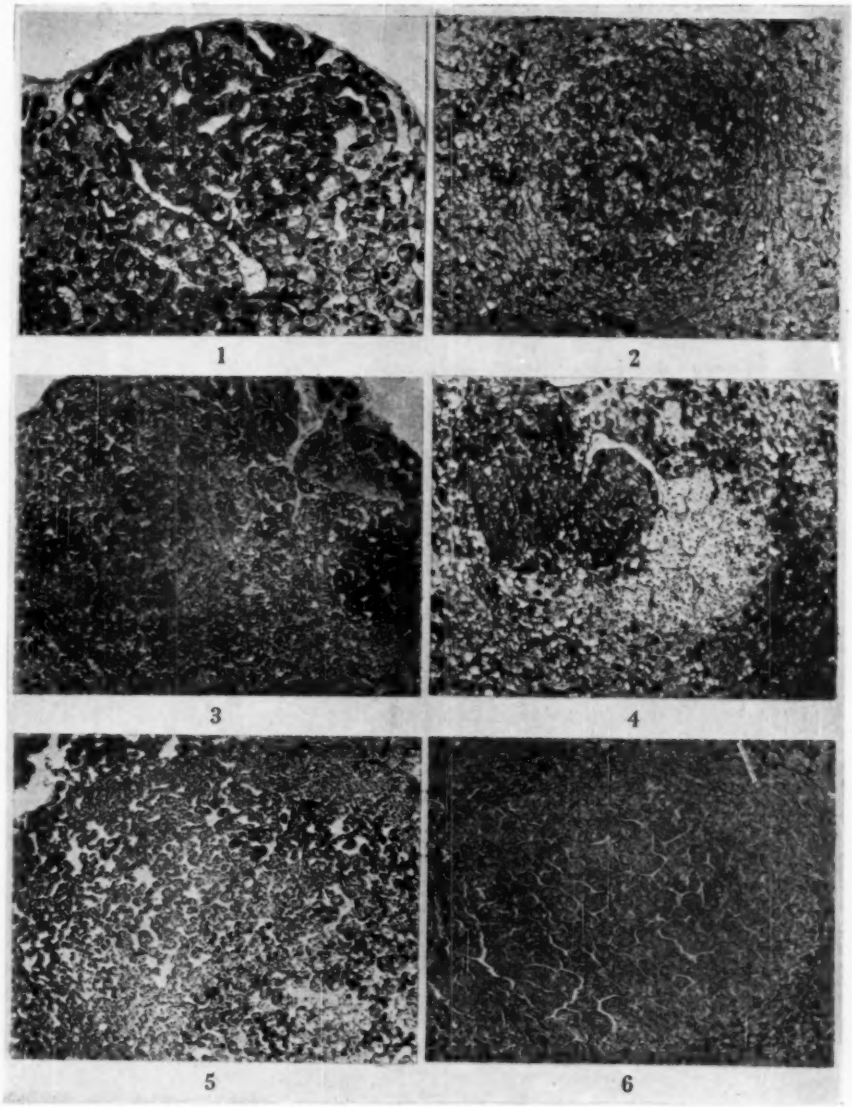
FIG. 12.—High power view of part of the adenoma shown in Fig. 4. The region shown includes cells resembling central gonadotrophs in the left of the field and, to the right, cells of low-granule content. The dark spots in these cells are due to PAS staining of the cytoplasm within the Golgi region. PAS × 400.

FIG. 13.—High power view of a basophil adenoma in the pars anterior of a rat killed 24 months after gonadectomy, showing cells with a marked resemblance to unhyalinised peripheral gonadotrophs. PAS × 750.

FIG. 14.—High power view of the basophil adenoma of Fig. 5 showing the resemblance of the cells to unhyalinised central gonadotrophs. PAS × 750.

FIG. 15.—High power view of a basophil adenoma in the pars anterior of a rat killed 24 months after gonadectomy. The cells are variable. A few are strongly granulated. Many are lightly granulated rounded cells resembling central gonadotrophs. Others have pale peripheral cytoplasm with a strong PAS reaction within the Golgi region. PAS × 330.

FIG. 16.—High power view of a basophil adenoma in a rat killed 24 months after gonadectomy. The cells are lightly granulated with the PAS reaction confined, in the main, to the Golgi regions which appear as black dots in the photomicrograph. PAS × 330.



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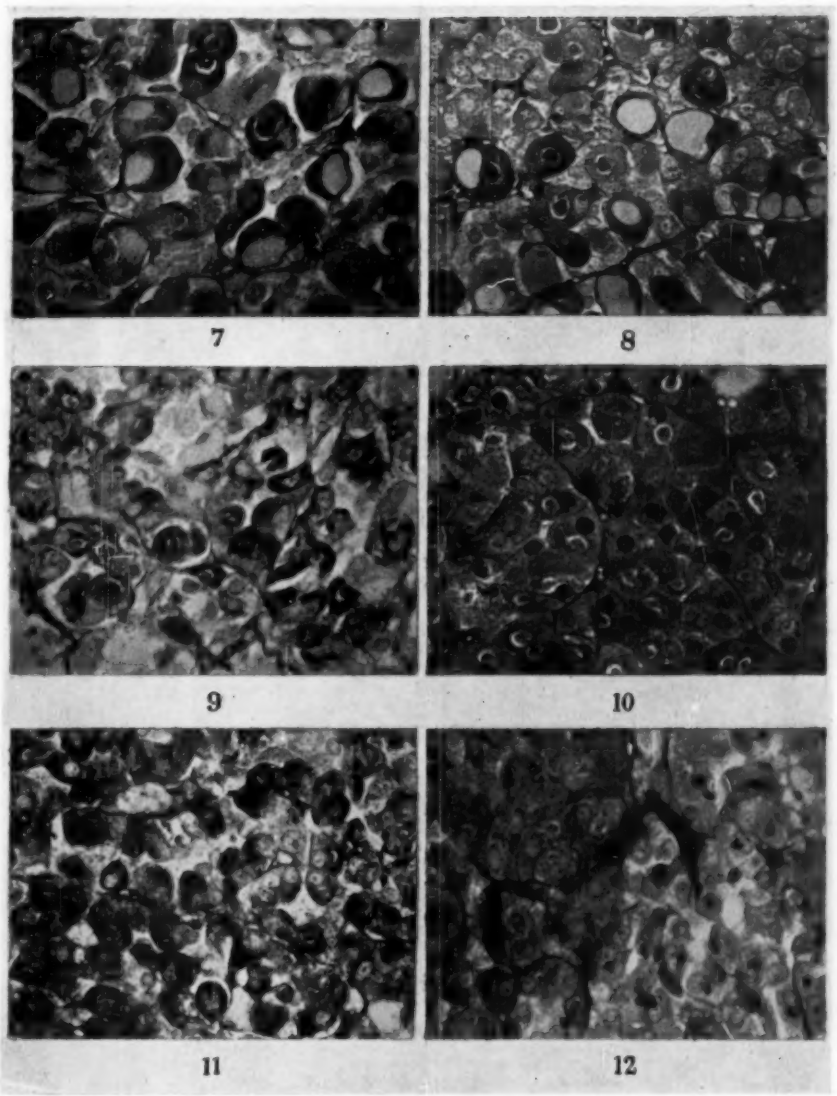

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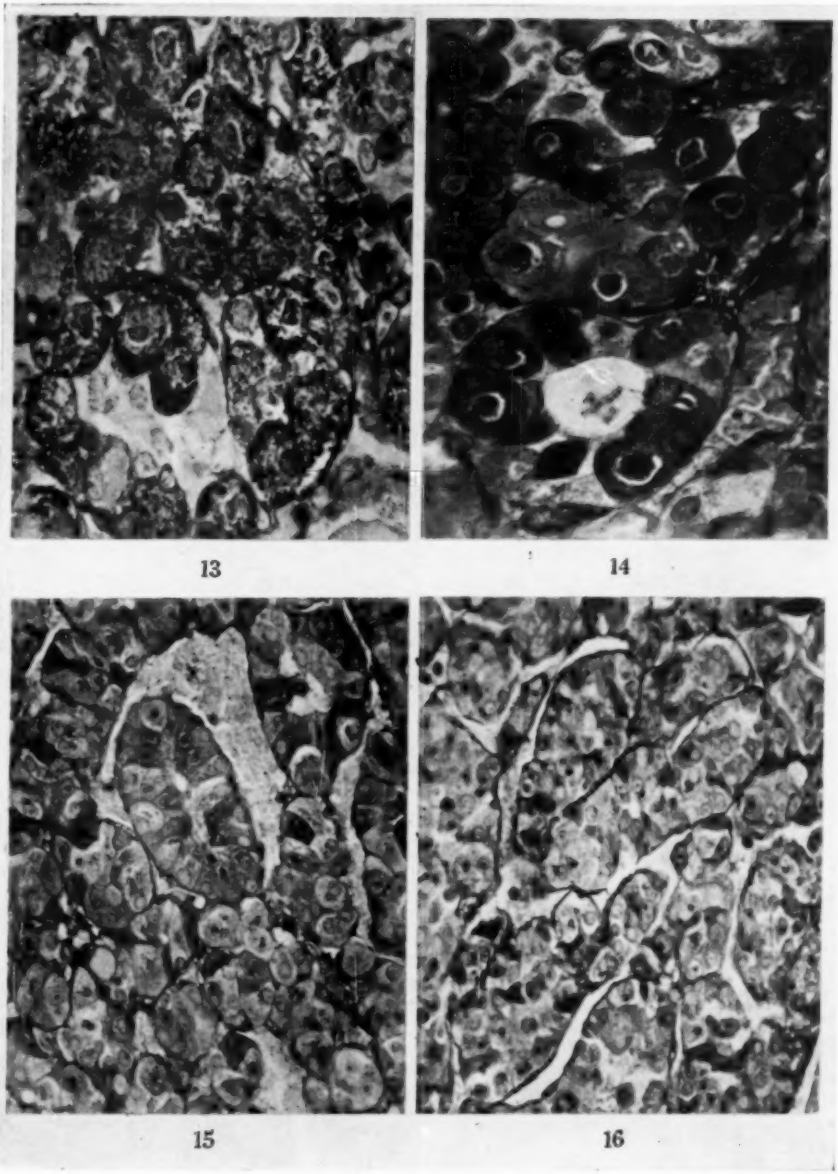

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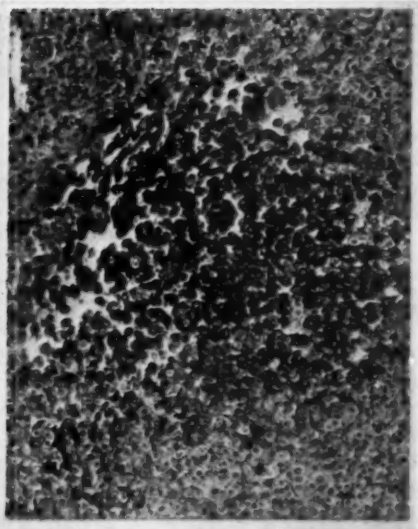

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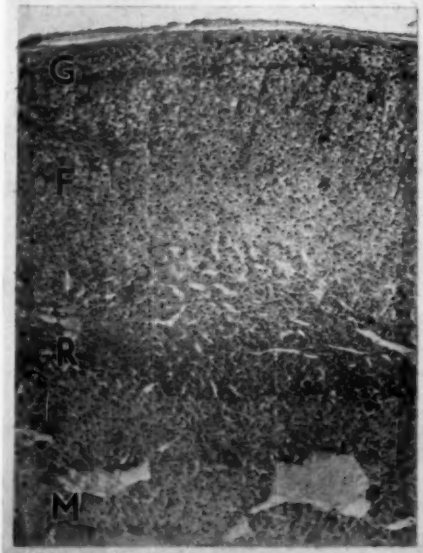

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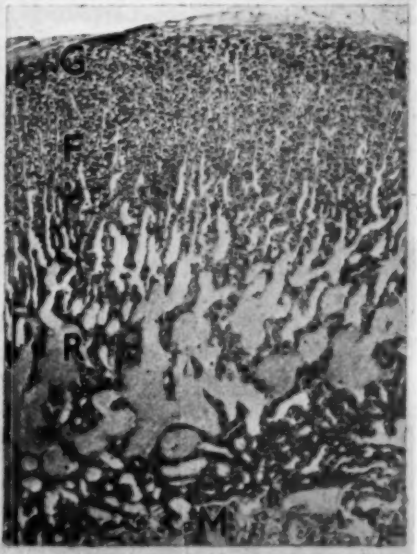

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therefore assumed that oestrogens or androgens of adrenocortical origin were implicated in the formation of the hypophysial adenomata. Dickie and Lane (1956), however, found in one strain of inbred mice that hypophysial adenomata appeared earlier and in higher incidence than adrenal neoplasms. We have tried to approach this problem by adrenalectomising our rats in combination with gonadectomy. The attempt to keep adrenalectomised rats for the long-time interval, necessary for hypophysial adenoma formation, has failed through high mortality as well as through regrowth of cortical adrenal tissue. It was observed, however, that regrown adrenal tissue never exceeded the size of the normal adrenal, that it was usually found on one side only and that it never contained neoplastic structures as judged by microscopical examination. We are certain that in the surviving twenty-two adrenalectomised and gonadectomised rats no adrenal tumour can have influenced the formation of hypophysial adenomata. Hypophysial adenomata were found in these animals in nearly the same number (1·8 adenomata per hypophysis) as in the rats only gonadectomised (2·2 adenomata per hypophysis). The supply of minute amounts of oestrogens or androgens by adrenal cortical tissue could not be excluded in our adrenalectomy series. The fact should be stressed, however, that not a single animal of the 142, gonadectomised for 18–32 months, showed signs of stimulation of either the uterus or the seminal vesicles and prostate. The secondary sex organs without exception were atrophic. Bielschowsky (private communication) working with the same strain of rats has made the same negative observation. Nevertheless, we have investigated the influence of small doses of stilboestrol in pellet form on the production of pituitary adenomata. In gonadectomised animals the oestrogen did not accelerate the formation of tumours or increase the number found. The higher dose of oestrogen (0·25 mg. stilboestrol) may have stimulated the formation of the acidophil-chromophobe type of tumour in intact rats since the incidence of this type of tumour was significantly higher in Group F than in the untreated animals of Group K ($P < \cdot 01$).

On the evidence existing at the present time we do not believe that in gonadectomised rats sex hormones are indispensable for the initiation of hypophysial adenomata, although the possibility of oestrogen production in adrenocortical tumours in some strains of mice and rats is not denied (Woolley 1958).

It does not appear that the age of the animal at the time of gonadectomy affects in any way the subsequent course of hypophysial changes resulting eventually in delta cell tumour formation. In an earlier study (Purves and Griesbach, 1955) we concluded from the examination of a small number of

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FIG. 17.—Low power view of the pars anterior of a rat killed 17 months after gonadectomy. In the upper half of the field is a delta cell adenoma, the granulation of which is unstained. In the lower half of the field is a beta cell adenoma, the granulation of which is stained by aldehyde-fuchsin. AF.—Orange G. × 72.

FIG. 18.—Low power view of the pars anterior of a rat killed 19 months after gonadectomy, showing an acidophil adenoma composed of well-granulated acidophil cells. Mall. × 72.

FIG. 19.—Low power view of the adrenal cortex of a normal rat for comparison with Fig. 20. G, zona glomerulosa; F, zona fasciculata; R, zona reticularis; M, medulla. H. & E. × 72.

FIG. 20.—Low power view of the adrenal cortex of a rat killed 19 months after gonadectomy, showing changes seen in all long-term gonadectomised rats in this series. Note the widely dilated blood-vessels in the zona reticularis and the inner part of the zona fasciculata. H. & E. × 72.

gonadectomised rats that the stimulation of the gonadotrophs by gonadectomy continued at a high level for only four to six months and that, thereafter, the activity of these cells diminished. It, therefore, seemed that some additional factor, such as secretion from adrenal tumours, might be necessary for long-continued stimulation of gonadotrophs and eventual tumour formation after gonadectomy. We now believe that no factor other than gonadal hormone deficiency is necessary for these effects. The regression of the castration changes, which we previously observed after long-term gonadectomy, we now ascribe to the effects of intercurrent infection, particularly to a chronic lung disease prevalent in our rat colony at that time. Early in the present study we noticed that rats which had been unhealthy for some time before death would show a regression or an absence of castration changes in the hypophysis, while in healthy members of the same group the castration changes would be present at full intensity.

The acidophil-chromophobe group of tumours included a few with well-granulated acidophil cells which would be easily recognised as an acidophil tumour. In most of these tumours only small amounts of granulation were present. The tumours, therefore, were weakly stained and appeared pale and relatively chromophobic as seen at low magnification. Under high power examination the presence of small numbers of acidophil granules in many of the tumour cells could be seen and occasional enlarged cells of high granule content were found. The presence of these granules characterises the cells of these tumours as belonging to the acidophil class—i.e., as being cells the storage form of whose secretion, when present, is the acidophil granule. We have called these tumours acidophil-chromophobe to indicate that while the tumours may be predominantly chromophobic they are composed of lightly granulated and degranulated cells of the acidophil class. The somewhat greater incidence of acidophil-chromophobe tumours in gonadectomised animals (34 per cent), as compared with the non-gonadectomised groups (22·9 per cent) cannot be regarded as significant. It appears that these tumours occur spontaneously in old animals, and the greater incidence in the gonadectomised rats might be ascribed to a greater average age in these animals. It is, however, noteworthy that gonadectomy does not hinder the appearance of tumours of this type, nor does it prevent lactogenic hormones being secreted in amounts which have striking effects on the mammary gland. It appears that mammary development and lactation are indeed much more likely to occur in gonadectomised animals bearing such tumours than in non-gonadectomised animals. Although not seen in this series, mammary growth and lactation can occur in intact animals bearing spontaneous acidophil-chromophobe tumours, one such case occurring in a female rat having been observed in our colony.

SUMMARY

A high incidence of basophil adenomata was found in the hypophyses of rats of both sexes examined 15 months or more after gonadectomy. No factor other than a long-continued deficiency of gonadal hormones in an otherwise healthy rat was necessary for the induction of adenomata.

The cells of basophil adenomata induced by gonadectomy had the staining reactions of delta cells and in most tumours resembled in cytological features the normal gonadotrophic basophils from which they were evidently derived.

Functioning acidophil tumours secreting lactogenic hormone were frequent in

gonadectomised animals. Similar manifestations of lactogenic hormone secretion were much less common in old, normal animals bearing acidophil tumours.

REFERENCES

BARRNETT, R. J., LADMAN, A. J. AND MCALLASTER, N. J.—(1955) *J. Histochem. Cytochem.*, **3**, 391.

BIELSCHOWSKY, F.—(1955) *Brit. J. Cancer*, **9**, 80.

CROSSMON, G.—(1937) *Anat. Rec.*, **69**, 33.

DICKIE, M. M. AND LANE, P. W.—(1956) *Cancer Res.*, **16**, 49.

GABE, M.—(1953) *Bull. Micr.*, appl., **3**, 152.

GOMORI, G.—(1950) *Amer. J. clin. Path.*, **20**, 665.

GRIESBACH, W. E. AND PURVES, H. D.—(1956) *Proc. Univ. Otago med. Sch.*, **34**, 1.

HALMI, N. S.—(1950) *Endocrinology*, **47**, 289.

HERLANT, M.—(1956) *Arch. Biol., Liège*, **67**, 89.

HOUSSAY, B. A., HOUSSAY, A. B., CARDEZA, A. AND PINTO, R. M.—(1955) *Schweiz med. Wschr.*, **85**, 291.

PURVES, H. D.—(1956) *Proc. R. Soc. Med.*, **49**, 1014.

Idem AND GRIESBACH, W. E.—(1951*a*) *Endocrinology*, **49**, 244.—(1951*b*) *Ibid.*, **49**, 427.—(1951*c*) *Ibid.*, **49**, 652.—(1954) *Ibid.*, **55**, 785.—(1955) *Ibid.*, **56**, 374.

WOOLLEY, G. W. AND DICKIE, M. M.—(1949) *Cancer Res.*, **9**, 373.

WOOLLEY, G. W.—(1958) *Ciba Colloquia on Endocrinology*, **12**, 122. London (J. & A. Churchill).

HISTOLOGICAL AND HISTOCHEMICAL CHANGES INDUCED BY ALKYLATING AGENTS IN TRANSPLANTED RAT SARCOMA WITH SPECIAL REFERENCE TO SARCOLYSINE

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SINCE substances with antineoplastic activity became available, particularly those known as biological alkylating agents, systematic histological and cytological studies of their action have been made (Yoshida, 1952; Jdanov, 1955; Koller and Veronesi, 1956; Kellner, 1957; Breuvis, 1958; Koller, 1958; Larionov and Presnov, 1958; Presnov and Spasskaya, 1959).

In this report the histological and histochemical changes that occur in several experimental tumours following the administration of particular alkylating agents are described.

MATERIALS AND METHODS

Three transplanted rat tumours were used.

Sarcoma 45.—This is a rat tumour of Russian origin. It is a malignant spindle-cell tumour, transplantation of which is successful in 100 per cent of animals, and which kills its host in 25 to 30 days. This tumour regresses completely with sarcolysine, dopan and ethylene-imino-benzoquinone (E39).

Sarcoma M-1.—This is a malignant, polymorphous-cell tumour of Russian origin. On transplantation 100 per cent of grafts are successful, and the tumour kills its host in 30 days. Sarcoma M-1 is more resistant to chemotherapy than Sarcoma 45.

Yoshida sarcoma.—This well-known tumour was first introduced into the Soviet Union in 1957.

All three tumours were transplanted subcutaneously into albino rats of either sex weighing between 80 and 100 g. The Yoshida sarcoma was also studied in the ascites form. 633 rats were used.

Chemotherapy was begun when the transplanted tumours were 7 to 10 days old, measured 0·7 to 1·0 cm. in diameter, and weighed 0·5 to 1·0 g. In the case of the ascites tumour, treatment was begun 4 days after transplantation.

Three alkylating agents were used: sarcolysine, *p*-di-(2-chloroethyl)amino-DL-phenylalanine, (Larionov, Khokhlov, Schkodinskaya, Vasina, Truscheikina, Novikova-Smirnova, 1955; Truscheikina, 1956; Larionov, 1959) known as merphalan in Great Britain (Bergel and Stock, 1953, 1954; Bergel, Burnop and Stock, 1955); dopan, 5-di-(2-chloroethyl)amino-4-methyl-uracil (Larionov and Platonova, 1955; Platonova, 1957; Larionov, 1959); ethylene-imino-benzoquinone, E.39, 2, 5-diethylenimino-3,6-dipropoxy-1,4-benzoquinone (Domagk, Peterson, and Gauss, 1954; Domagk, 1958).

Rats bearing Sarcoma 45 received either sarcolysine or dopan ; those bearing Sarcoma M-1 received only sarcolysine, while rats bearing the Yoshida tumour received each of the three compounds. The doses and routes of administration of the drugs are shown in Table I.

TABLE I.—*Doses and Routes of Administration of Alkylating Agents in Transplated Rat Tumours.*

Tumour	Sarcolysine	Dopan	E39
Sarcoma M-1	Intraperitoneal 5 mg. per kg. 5 to 7 times, 3-day intervals 10 mg. per kg. 5 to 7 times at 3-* or 7-day intervals	—	—
Sarcoma 45	15 mg. per kg. once or in 3–5 divided doses 5 mg. per kg. at 3-day intervals	Oral 0·3 mg. per kg. daily for 15 days, or 0·75 mg. per kg. 7–9 times every 3 days	—
Yoshida solid	Intraperitoneal 1·5 mg. per kg. daily for 20 days	Oral 0·3 mg. per kg. daily for 20 days	Intraperitoneal 0·6 mg. per kg. daily for 20 days.
Yoshida ascites	As above, or orally	—	—

* Some rats died.

The histological and histochemical methods used were as follows :

(a) Paraffin-embedded material fixed in 10 per cent neutral formol saline. Haematoxylin and eosin, Feulgen's method for deoxyribonucleic acid, methyl-green-pyronin for ribonucleic acid, Van Gieson's stain for collagen, Foot's stain for reticulin, and toluidine blue for metachromasia.

(b) Frozen sections of material fixed in 10 per cent neutral formol saline. Sudan III for neutral fat.

(c) Paraffin-embedded material fixed in cold 80 per cent ethanol. Gomori's method for alkaline phosphatase. The incubation time was between 2 and 5 hours (Presnov, 1954, 1956).

(d) Paraffin-embedded material fixed by Schabadasch's method (1949). McManus's modification of Hotchkiss's periodic-acid-Schiff (PAS) method for polysaccharide.

(e) Methanol-fixed smears of ascitic fluid. May-Grünwald-Giemsa.

(f) Vital fluorescence microscopy was carried out in collaboration with Dr. P. V. Breuvis to study the nucleoproteins.

To allow for artefacts in staining, the treated and untreated animals were killed at the same time, and the tissues from both were handled side by side throughout each procedure, so that every preparation from a treated tumour could be compared directly with its counterpart from an untreated tumour which had been subjected to identical manipulation.

RESULTS

Sarcoma 45 regressed in every animal under treatment with sarcolysine and dopan, but Sarcoma M-1 regressed completely only after administration of

sarcolysine at high dosage (five to seven injections at 3-day intervals of 10 mg. per kg. of body weight). Subcutaneous nodules of the Yoshida sarcoma regressed completely under therapy with sarcolysine or dopan in only 25 per cent of the rats, and in only 12 per cent when E39 was given. Sarcolysine always caused complete regression in the ascitic form of Yoshida sarcoma, whether oral or intraperitoneal routes were employed.

The histological and histochemical changes during the course of regression were similar in all three tumours and with each of the three drugs.

General histological changes.—During the course of regression, striking changes were observed both in the parenchyma and stroma of the tumours. The earliest changes, decrease in mitotic activity and nuclear fragmentation, were observed within 1 hour of the intraperitoneal injection of sarcolysine in the case of the Yoshida ascites sarcoma, and in 3 hours in the case of the solid sarcomata, or in the ascites sarcoma after oral administration. Mitotic activity disappeared if treatment was continued. Cells in prophase were the first to disappear. Cell destruction accompanied the decrease in mitotic activity, their nuclei became angular and the chromatin coarsened. Pyknotic changes were less common. Abnormal multipolar mitoses appeared. This sequence of changes is illustrated in Fig. 1–6, 16–24. A very characteristic change was the enlargment of many of the cells, which has been frequently described as a feature of the action of alkylating agents. With further decrease in mitotic activity, bizarre multinucleated giant cells appeared, presumably as a result of amitotic division (Fig. 2, 6, 17, 18). During treatment the nucleoli of the tumour cells increased in size, showed increased pyroninophilia and alkaline phosphatase activity.

The stromal and parenchymal changes described did not occur simultaneously throughout the tumour but were patchy and irregular. In the Yoshida ascites cells the changes were comparable with those seen in the solid tumours, and included the appearance of pathological mitoses, the formation of multinucleated cells and marked enlargement of the cells and their nuclei and nucleoli (Fig. 22–24).

Changes in the nucleoproteins.—The first change observed was droplet formation within the nuclei (Fig. 5). The droplets stained deeply with Feulgen's stain and also showed alkaline phosphatase activity. The nuclei of the greatly enlarged cells were Feulgen negative almost always, but rarely gave a positive reaction.

Studies on the nucleoproteins in this material, by means of vital fluoresence microscopy showed that changes occurred 3 to 6 hours after the first treatment with sarcolysine, and consisted of increased fluoresence in the nuclei of affected cells (Breuvis, 1958).

Fatty change.—Fat is seen only in small amounts in necrotic areas of the untreated tumours, but appeared in the form of small droplets in the cytoplasm of a majority of the cells 24 to 48 hours after the beginning of treatment. Later all the cells contained fat, and the small droplets fused into larger ones, becoming liberated in the stroma as cells disintegrated. Eventually large quantities of fat accumulated, and this could still be recognised embedded in the scar which remained after the tumour had regressed completely and had become replaced by fibrous tissue.

Alkaline phosphatase activity.—In untreated tumours, the enzyme is located mainly in the nuclei and nucleoli of the cells. Following treatment, it appeared also in the cytoplasm, while in fragmented nuclei the amount increased greatly.

Later alkaline phosphatase activity appeared also in the stroma and between the newly formed collagen fibres (Fig. 7, 8. 9).

Ribonucleic acid.—Pyroninophilia, sometimes after an initial increase, gradually decreased in the tumour cells, and later disappeared entirely.

Metachromasia.—Cytoplasmic metachromasia with toluidine blue also disappeared during treatment.

Glycogen.—Glycogen is not present in untreated tumours, except in lymphocytes in the stroma, or in necrotic areas. Glycogen did not appear in the tumour cells as a result of treatment, though pink droplets, not disappearing after treatment with amylase, appeared (McManus-Hotchkiss reaction). They were presumably mucopolysaccharide in nature.

Stromal reaction.—The first change was oedema, which separated the tumour cells slightly from one another. Untreated tumours contain very little collagen, but argyrophilic fibres are numerous. During treatment, collagen fibres appeared, became increasingly numerous and eventually formed, a dense scar. During the same period, the argyrophilic fibres lost their affinity for silver stains, became thicker and were presumably transformed into collagen fibres (Fig. 10–15).

DISCUSSION

Alkylating agents kill cells. Death may be rapid, and is shown by fragmentation of the nuclei, or slow, when death is preceded by the appearance of fat in the cytoplasm, increase in alkaline phosphatase activity, disappearance of ribonucleic acid and of chromotropic substances from the cytoplasm, and by enlargement of the nuclei and nucleoli. Amitotic division may be a form of dystrophic change in which the cells, though damaged, can survive. Since large doses of alkylating agents can cause complete regression of tumours, it is likely that all the cells are damaged to some extent, and the type and degree of damage may depend on the functional activity of the cell at the time of exposure, some states of activity rendering the cell more vulnerable than others. The drugs studied appear to affect the process of cell division and also to interfere with growth, the latter effect being relatively small. Sarcolysine appears to suppress the growth of cells more than dopan or E39.

The alkylating agents appear to act on the tumour cells immediately as shown by the rapidity with which visible changes in the cells occur, for instance in the ascites tumours. That nucleoproteins are damaged directly is suggested by the early occurrence of nuclear fragmentation, increased fluorescence and decrease of mitotic activity. It is possible that the exact mechanisms vary from one alkylating agent to another. Powerful agents like sarcolysine might have additional actions.

It is important to emphasize that the stromal reaction follows the changes in the tumour cells, and is perhaps directed towards removal of the damaged tumour cells. It is assumed that increased alkaline phosphatase activity helps in removing fragmented cell components rich in phosphoesters and nucleic acids.

SUMMARY

1. The histological and histochemical changes accompanying drug-induced regression were followed in three transplanted rat tumours.

2. The tumours were the Sarcoma 45, the Sarcoma M-1, and the Yoshida sarcoma in solid and ascites form.

3. The drugs used were the alkylating agents sarcolysine (merphalan), dopan, and ethylene-imino-benzoquinone (E39).

4. Eleven histological and histochemical methods were used.

5. Striking parenchymal and stromal changes were observed. Early changes included decrease in mitotic activity and nuclear fragmentation, while later, enlargement of surviving cells, and the appearance of multinucleated forms was seen. Fat appeared in the cytoplasm, and ribonucleoprotein and chromotropic substances disappeared. In the stroma, the argyrophilic fibres became converted into collagen. Alkaline phosphatase activity increases first in the cells, and later in the stroma.

6. The alkylating agents appear to act immediately on the tumour cells. The stromal reaction is secondary to the death of the tumour cells. The drugs affect the nucleoproteins directly.

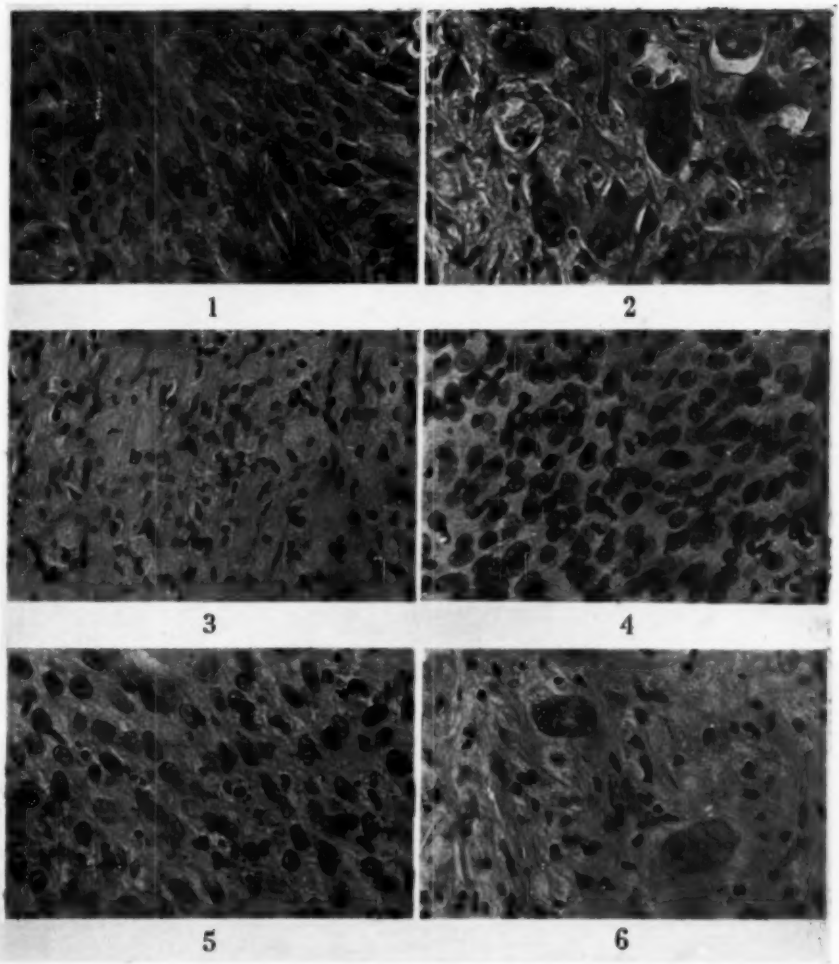
I wish to thank Professor L. F. Larionov for helpful advice.

REFERENCES

BERGEL, F. AND STOCK, J. A.—(1953) *Rep. Brit. Emp. Cancer Campgn.*, **31**, 6.—(1954) *J. chem. Soc.*, 2409.

EXPLANATION OF PLATES

FIG. 1.—Sarcoma 45. Untreated tumour. Haematoxylin and eosin. ×280.
FIG. 2.—Sarcoma 45. 24 hours after the 3rd intraperitoneal injection of sarcolysine, 5 mg. per kg. of body weight at 72-hour intervals. Haematoxylin and eosin. ×280.
FIG. 3.—Sarcoma 45. 72 hours after the 3rd injection. Haematoxylin and eosin. ×280.
FIG. 4.—Sarcoma 45. Untreated. Feulgen reaction. ×280.
FIG. 5.—Sarcoma 45. 48 hours after sarcolysine, 5 mg. per kg. of body weight. Feulgen reaction. ×280.
FIG. 6.—Sarcoma 45. 24 hours after the 3rd intraperitoneal injection of sarcolysine, 5 mg. per kg. of body weight at 72-hour intervals. Feulgen reaction. ×280.
FIG. 7.—Sarcoma 45. Untreated. Gomori reaction for alkaline phosphatase. ×280.
FIG. 8. Sarcoma 45. 24 hours after the 2nd intraperitoneal injection of sarcolysine. Gomori reaction. ×280.
FIG. 9.—Sarcoma 45. 72 hours after the 3rd injection. Gomori reaction. ×280.
FIG. 10.—Sarcoma 45. Untreated. Van Gieson. ×280.
FIG. 11.—Sarcoma 45. 72 hours after the 2nd intraperitoneal injection of sarcolysine. Van Gieson. ×280.
FIG. 12.—Sarcoma 45. 72 hours after the 3rd intraperitoneal injection. Van Gieson. ×280.
FIG. 13.—Sarcoma 45. Untreated. Foot. ×280.
FIG. 14.—Sarcoma 45. 24 hours after the 3rd intraperitoneal injection of sarcolysine. Foot. ×280.
FIG. 15.—Sarcoma 45. 72 hours after the 3rd intraperitoneal injection. Foot. ×280.
FIG. 16.—Sarcoma M-1. Untreated. Haematoxylin and eosin. ×280.
FIG. 17.—Sarcoma M-1. 48 hours after 2 injections of sarcolysine, 10 mg. per kg. of body weight at 73-hour intervals. Haematoxylin and eosin. ×280.
FIG. 18.—Sarcoma M-1. 48 hours after 5th injection of sarcolysine, 10 mg. per kg. of body weight at 72-hour intervals. Haematoxylin and eosin. ×280.
FIG. 19.—Sarcoma M-1. Untreated. Feulgen. ×280.
FIG. 20.—Sarcoma M-1. 48 hours after the 3rd injection of sarcolysine. Feulgen. ×280.
FIG. 21.—Sarcoma M-1. 48 hours after the 5th injection. Feulgen. ×280.
FIG. 22.—Yoshida ascites tumour. Untreated. May-Grünwald-Giemsa. ×630.
FIG. 23.—Yoshida ascites tumour. 24 hours after the intraperitoneal injection of sarcolysine, 1·5 mg. per kg. of body weight. May-Grünwald-Giemsa. ×630.
FIG. 24.—Yoshida ascites tumour. 24 hours after 3 daily intraperitoneal injections of sarcolysine, 1·5 mg. per kg. of body weight. May-Grünwald-Giemsa. ×630.



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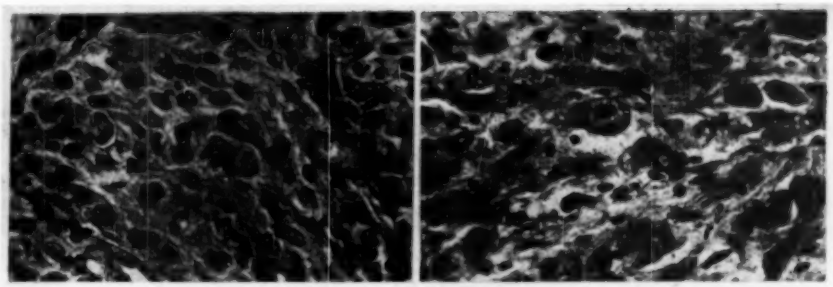

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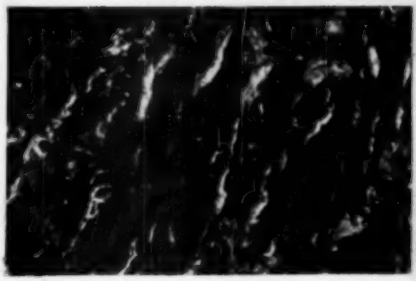

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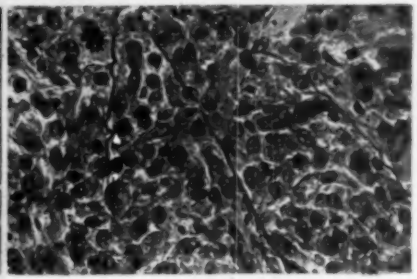

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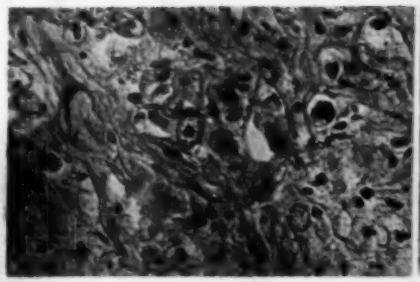

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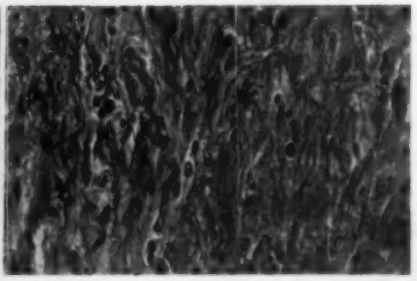

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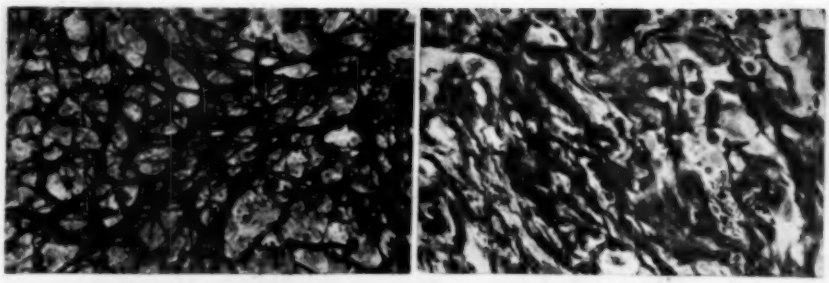

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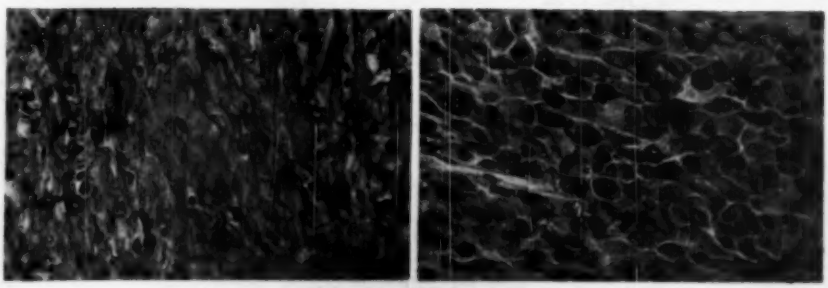

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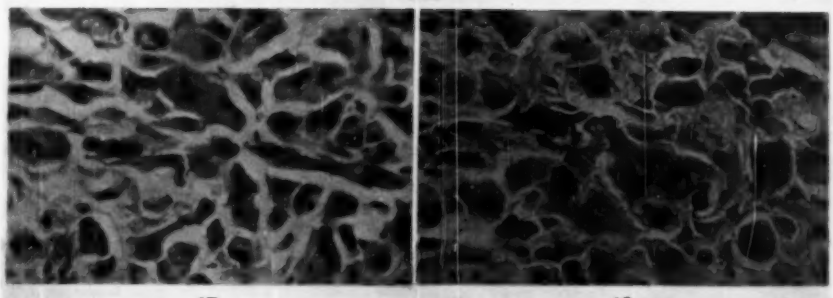

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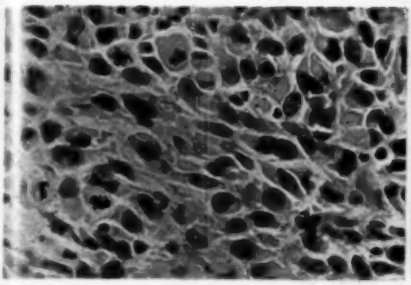

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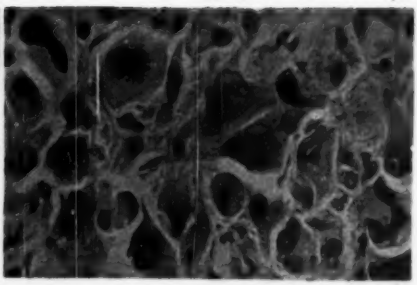

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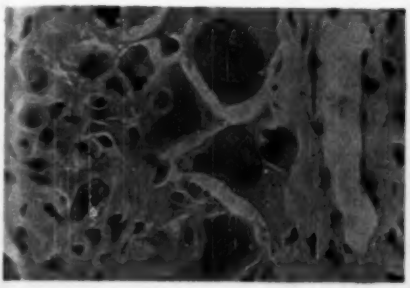

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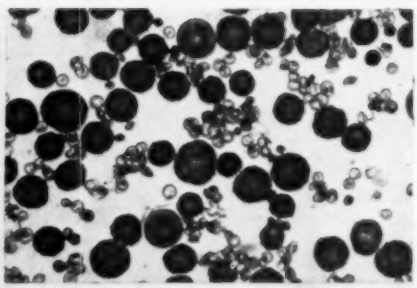

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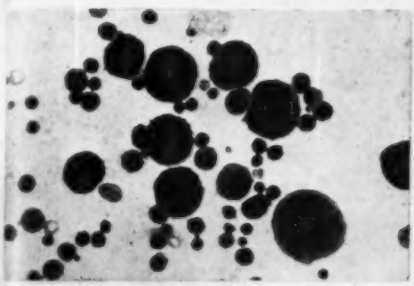

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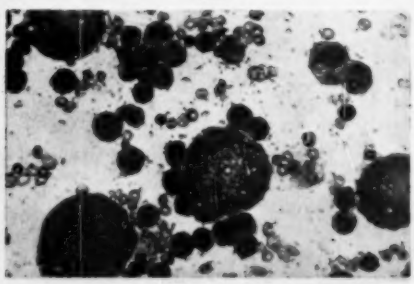

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Presnov.

Idem, BURNOP, V. C. E. AND STOCK, J. A.—(1955) *Ibid.*, 1223.
BREUVIS, P. V.—(1958) *Arkh. Pat.*, **20**, 39.
DOMAGK, G.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 1197.
Idem, PETERSON, S. AND GAUSS, W. A.—(1954) *Z. Krebsforsch.*, **59**, 617.
KELLNER, B.—(1957) *Acta Morph. Acad. Sci., Hung.*, **7**, 4.
KOLLER, P. C.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 783.
Idem AND VERONESI, U.—(1956) *Brit. J. Cancer*, **10**, 703.
JDANOV, G. L.—(1955) *Probl. Oncol.*, **6**, 94.
LARIONOV, L. F.—(1959) *Acta Un. int. Cancr.*, **15**, 42.
Idem, KHOKHLOV, A. S., SCHKODINSKAYA, E. N., VASINA, O. S., TRUSCHEIKINA, V. I., NOVIKOVA-SMIRNOVA, M. A.—(1955) *Bull. exp. Biol. Med.*, **1**, 48.
Idem AND PLATONOVA, G. N. (1955) *Probl. Oncol.*, **5**, 36.
Idem AND PRESNOV, M. A.—(1958) *Arkh. Pat.*, **20**, 32.
PLATONOVA, G. N.—(1957) *Bull. exp. Biol. Med.*, **6**, 53.
PRESNOV, M. A.—(1954) *J. gen. Biol.*, **5**, 321.—(1956) *Probl. Oncol.*, **4**, 423.
Idem AND SPASSKAYA, I. G.—(1959) *Ibid.*, **7**, 38.
SCHABADASCH, A. L.—(1949) ' Problems Connected with the Histochemical Investigation of Glycogen in the Normal Nervous System. Study on the Biological Characteristic of and Differences Between Neurones '. Moscow (Medgiz).
TRUSCHEIKINA, V. I.—(1956) *Probl. Oncol.*, **2**, 222.
YOSHIDA, T.—(1952) *J. nat. Cancer Inst.*, **12**, 947.

GRAFT-VERSUS-HOST REACTIONS IN THE RABBIT

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UNTIL recently the homograft reaction was mainly studied from the aspect of rejection of the foreign tissue transplant by its host. Yet, as Medawar (1958) has pointed out, grafting is an act of parabiosis, however unequal the partners to the union may be, and the possibility of the transplant mounting a counter-attack against the recipient must be considered.

It was the work of Dempster (1953) and Simonsen (1953) on kidney homotransplants in dogs that first indicated that reactions of this nature might be more than a theoretical hazard. Since then the grave consequences of graft intolerance of the host have been recognised following induction of tolerance in newborn animals by the injection of adult spleen cells (Billingham and Brent, 1957; Simonsen, 1957), following treatment of lethally X-irradiated animals with homologous bone marrow (Trentin, 1956; Congdon and Urso 1957). and following injection of F1 hybrid mice with adult spleen cells from the parental strains (Cole and Ellis, 1958; Trentin, 1958; Gorer and Boyse, 1959). In all these instances the injected mice are for one reason or another unable to destroy the introduced foreign lymphoid cells which then proliferate and proceed to assail the host cells.

Although it is generally agreed that the victims lose weight and characteristically show atrophy of their lymphoid tissue, few detailed descriptions of the pathological appearances associated with these syndromes are available.

The purpose of this report therefore is to describe and compare the histological changes seen in rabbits suffering from secondary irradiation disease and runt disease.

MATERIALS AND METHODS

Animals.—Young adult Chinchilla rabbits which were not inbred in the genetic sense were used throughout this study.

Production of radiation-chimaeras.—Lethally X-irradiated rabbits were treated intravenously with bone marrow cells from normal adult rabbits.

1. *X-irradiation.*—Whole body X-irradiation was given as a horizontal beam to 2 animals at a time from a Westinghouse machine under the following conditions: 220 kV., 12·5 mA., 70 cm. target to skin distance, 1·0 mm. Cu and 1·0 mm. Al filters, H.V.L. 1·8 mm. Cu, dose rate to skin 33·4 r per minute, to centre of animal 21·1 r per minute. The dosage was divided; an initial dose of 600 r at the centre of the animal was followed 24 hours later by 500 r, and after another 24 hours by a further 500 r, giving a total of 1600 r (L.D. 100/30 days).

2. *Bone marrow.*—A suspension of adult homologous bone marrow was prepared from a female rabbit as described earlier (Porter and Murray, 1958), and 1200 ($\times 10^6$) nucleated cells injected into the marginal ear vein of an irradiated

male rabbit 1–3 hours following the last dose of X-rays, and within 1 hour of the death of the female donor.

3. *Criterion for success.*—The successful establishment of a radiation-chimaera was accepted only if female type heterophils (amphophils) appeared in the irradiated male rabbit (Porter, 1957*a*).

Induction of immunological tolerance

Spleen cells from adult homologous female rabbits were injected into foetal rabbits (Porter, 1960*a*).

1. *Preparation of spleen cell suspensions.*—A mature female rabbit weighing 2·5–3·0 kg. was anaesthetised with intravenous Nembutal and a splenectomy performed through an upper midline abdominal incision. Immediately after removal the spleen was finely chopped under aseptic conditions in heparanized saline solution and then suspended by gently drawing up and down through a wide bore cannula. After filtration through nylon bolting cloth of 90 μ porosity a cell count was made and a suitable quantity of the spleen suspension containing 50 ($\times$ 10^6) nucleated cells injected into the recipient animal.

2. *Injection of foetal rabbits.*—A rabbit at the 20th or 22nd day of pregnancy (normal gestation period is 31 days), was anaesthetised with intravenous Nembutal and a midline abdominal incision made extending from the level of the last pair of nipples to just below the umbilicus. When the abdomen had been explored to determine the number of foetuses present, the free end of one horn of the uterus was delivered through the wound. The position of each foetus was easily seen and its head and back identified by palpation. Adult spleen tissue suspension was then injected through the wall of the uterus into the peritoneal cavity of each foetus. After returning the uterus to the abdomen by gentle steady pressure, the peritoneum and linea alba were sutured as one layer with 3–0 chromic gut. The skin was sutured separately.

3. *Skin grafts.*—When possible the recipients of spleen cell suspension were later challenged with skin grafts from the spleen donor. These were full thickness homografts 2 cm. in diameter sutured into prepared beds upon the ears as described by Stark, Brownlee and Grunwald (1958).

Weight.—The rabbits were weighed daily for the first month, then at weekly intervals.

Antibiotic therapy.—All irradiated rabbits were given tetracycline hydrochloride intramuscularly 50 mg. per day for the first 2 weeks following irradiation.

Pathology.—All animals that were killed or died were examined post mortem. A pencil of marrow was dissected from the right femur, the sample always being taken from a comparable position at the mid-point of the shaft. Most tissues were fixed in formol-saline, but the marrow, lymph nodes and spleen were fixed in Helly's fluid and all were routinely stained with haematoxylin and eosin. Special stains were used where necessary, and marrow smears were stained by Leishman's method.

Sex chromatin was also sought in lymphocytes obtained from the lymph nodes and spleen of some animals, using the method outlined by Riis (1957). The cell suspension to be examined was incubated for 24 hours in an homologous plasma coagulum and then appropriately fixed and stained. The disadvantages of this technique have already been discussed (Porter, 1960*b*).

Experimental procedure

In this experiment 4 groups of animals were studied.

Group I consisted of 5 normal adult non-irradiated rabbits and 14 baby rabbits of various ages. Animals from this group were killed at appropriate times for comparison with those that had died or been killed amongst the treated groups.

Group II was composed of 20 X-irradited rabbits given no subsequent treatment. They were examined histologically after death.

Group III consisted of 20 radiation-chimaeras with " secondary disease ".

In the accumulation of this group all successful radiation-chimaeras that were regaining the weight lost immediately after irradiation, were subjected to biopsy of an inguinal or axillary lymph node at 14 days after marrow treatment. After this time any animal that continued to show a constant percentage of polymorphs with female sex chromatin, and that was clinically free of infection, but started to lose weight again, was regarded as developing " secondary disease ". Taking the day wasting was first noticed as the beginning of " secondary disease ", groups of 3 animals were killed on the 1st, 7th, 14th and 21st day of the process for histological examination. The remainder were examined whenever they happened to die.

Group IV was composed of 20 non-irradiated tolerant rabbits with " runt disease ".

In allocating animals to this group those rabbits subjected to intra-uterine homologous spleen injection that failed to gain weight properly after birth, were considered to have " runt disease ". The majority died aged 2–6 weeks and were then examined histologically. In addition groups of 2 animals were killed and examined at intervals of 1, 2 and 3 weeks after birth.

RESULTS

Rabbits exposed to lethal X-irradiation without marrow treatment (Group II)

All rabbits in this group had died by the 10th day, the mean survival time being 8·1 $\pm$ 1·12 days. At post mortem some slight wasting was always present and the fur often appeared dull and tended to be shed easily.

Bone marrow.—There was destruction of practically all the haemopoietic cells of the femoral bone marrow, only the reticular cells surviving.

Spleen.—This organ was smaller than normal. The mean splenic weight being 0·22 $\pm$ 0·062 g. per kg. of body weight compared with 0·55 $\pm$ 0·046 g. for a group of 20 normal rabbits. The decrease in size was due to almost complete destruction of the lymphoid tissue. The reticular cells in the white pulp remained. The red pulp was often congested and always contained haemosiderin, much within macrophages.

Lymphoid tissue elsewhere.—The thymus showed destruction of cortical lymphocytes with shrinkage of the lobules and contraction of the stroma to form a solid sheet of cells in the region where the cortex had been. Similar destruction of lymphocytes with no evidence of regeneration was seen in the mesenteric, axillary and inguinal lymph nodes, and in the normal lymphoid collections in the gut, particularly the appendix. In 8 cases many red cells were present in the lymph node sinuses.

Respiratory system.—A necrotic and haemorrhagic non-purulent infection of the lungs by *Ps. pyocyaneus*, killed 5 of the animals in this group. Eight animals showed intra-alveolar haemorrhage without infection.

Gastro-intestinal tract.—Petechial haemorrhages into stomach and elsewhere in the gut were seen in 16 animals. Rupture of the fundus of the stomach caused the death of 4 animals and massive haemorrhage into the colon the death of 4 more. In 3 rabbits that died before 7 days there was a partial denudation of the intestinal mucosa ; the intervening areas consisting of flattened villi covered by stretched epithelial cells. In the remaining rabbits, regeneration was complete by the time they died from infection or haemorrhage.

Liver.—In 6 cases there was some atrophy of the liver cells and distension of the sinusoids with blood ; 5 more animals showed changes usually associated with chronic venous congestion.

Testes.—Active spermatogenesis had ceased.

No other characteristic lesions were noticed.

Radiation-chimaeras with " secondary disease " (Group III)

The animals with successful marrow transplants initially lost weight, but this was followed by recovery from about the 10th day after irradiation. However, in those developing " secondary disease ", at any time between the 16th and 40th days weight loss recurred, accompanied by early signs of diarrhoea. These symptoms became steadily more severe until the animal died. At post mortem the rabbits were invariably emaciated. Their fur was dull, dirty and shed easily.

Bone marrow.—All the animals, at whatever stage of " secondary disease " they were examined, showed a well-repopulated bone marrow (Fig. 1). Smears revealed the presence of " drumsticks " in some of the cells indicating that repopulation was from donor sources. Animals kil'ed or dying after the 14th day of " secondary disease " showed loss of normal fat and increased cellularity. The longer the disease had been present, the more pronounced this hyperplasia, which was predominately of the myeloid series. Plasma cells were rarely seen in the bone marrow. Erythropoiesis and megakaryocyte formation appeared to be normal. A few phagocytic cells containing haemosiderin were always to be seen. In 12 of the animals the stroma consisted of mucoid material which stained positively with mucicarmine.

Spleen.—First day of " secondary disease ". In the 3 animals killed at the beginning of " secondary disease ", the spleens appeared slightly larger than normal. Microscopically there was good repopulation of the white pulp with lymphocytes, immature plasma cells and some transitional cells, using these terms as defined by Fagraeus (1948). Mitotic figures were frequent, and small germinal centres were present in 2 of the animals. The red pulp contained about as much haemosiderin as was present in the control irradiated animals of Group II.

Seventh day of " secondary disease ". In animals killed at this stage the spleens were enlarged, the mean weight being 1·00 $\pm$ 0·183 g. per kg. of body weight (normal = 0·55 $\pm$ 0·046 g.), and microscopically the white pulp contained many transitional cells, immature plasma cells and a few mature plasma cells and lymphocytes (Fig. 2). Again mitotic figures were common. In 1 of the animals these cells were also found diffusely scattered throughout the red pulp. The congested red pulp contained plenty of haemosiderin, mostly in macrophages. Erythrophagocytosis was present in all 3 spleens.

Fourteenth day of " secondary disease ". The 3 rabbits killed at this time still showed slightly enlarged spleens with a mean weight of 0·58 ± 0·03 g. per kg. of body weight. Microscopically in some of the lymphoid nodules were cells with pyknotic or fragmented nuclei, and others which had undergone complete necrosis. Of the surviving cells a high proportion were mature plasma cells (Fig. 3). Other lymphoid nodules in the white pulp had already been severely depleted of cells and partly replaced by masses of smudgy fibrinoid material (Fig. 4). In one of the animals foreign body giant cells were associated with this fibrinoid substance. The red pulp was congested and now appeared to contain more haemosiderin than was present in the spleens of the control irradiated animals.

Twenty-first day of " secondary disease ". The spleens at this stage appeared shrunken with a mean weight of 0·29 ± 0·03 g. per kg. of body weight. Microscopically in 1 animal the white pulp had largely been replaced by immature collagen and numerous foreign body giant cells (Fig. 5). Lymphocytes, transitional cells and immature plasma cells had disappeared, but the red pulp contained scattered groups of mature plasma cells. Other animals examined at this time showed similar loss of lymphoid tissue and substitution of fibrous scars for Malpighian bodies, but without giant cells (Fig. 6). Scattered groups of mature plasma cells were, however, always present and in 2 animals small haemorrhages were also seen.

Two rabbits that died 30 and 34 days after the onset of " secondary disease " both showed extensive replacement of the depleted white pulp with mature collagen. In all instances the red pulp contained much haemosiderin.

Other lymphoid tissue.—When biopsied at 14 days after bone marrow treatment, the inguinal or axillary lymph nodes of those radiation-chimaeras which subsequently developed " secondary disease ", were small and showed early incomplete repopulation with lymphocytes (Fig. 7). In cell suspensions from a few of these lymph nodes female sex chromatin was demonstrated in lymphocytes by the method of Riis (1957).

By the time the first animals with " secondary disease " were killed the mesenteric and other lymph nodes were enlarged and showed an extensive proliferation of transitional cells and immature plasma cells, with relatively few mature plasma cells and lymphocytes. Lymphoid tissue elsewhere showed similar changes, but the thymus tended to lag in this process.

Little change was seen in the histological picture obtained at the 7th day of " secondary disease ", but by the 14th day many of the cells in the repopulated lymph nodes were undergoing necrosis (Fig. 8) and mature plasma cells were beginning to predominate amongst the surviving cells (Fig. 9). By the 21st day the lymph nodes were shrunken, depleted of lymphocytes, and only contained a sprinkling of mature plasma cells (Fig. 10). Lymphoid tissue elsewhere was similarly atrophied.

Respiratory system.—In 6 of the 8 radiation-chimaeras that perished from " secondary disease " the immediate cause of death was a patchy purulent peribronchiolar pneumonic consolidation. In 5 of these the organism responsible was *Ps. pyocyaneus*.

Gastro-intestinal tract.—Of the 20 radiation-chimaeras with " secondary disease " 4 animals showed discrete gastric ulceration. These chronic ulcers were about 0·5 cm. in diameter and situated at the pylorus. The high incidence of this lesion, often accompanied by perforation, is well recognised in the rabbit

after large doses of X-irradiation (Porter, 1957*b*). A colitis was present in 6 animals. This was most severe in the descending colon.

Liver.—Occasional focal areas of necrosis were present in the liver lobules of 4 of the 8 animals that died, and in those of 2 of the animals killed at 14 days and 1 of the animals killed at 21 days. These lesions were scattered throughout the organ and were not consistently found in any one zone of the liver lobule. They did not seem to be related to coccidial infection. Two of the 20 animals with " secondary disease " showed biliary cirrhosis. This lesion has been described previously (Porter, 1960*d*) and is usually associated with recrudescence of infection with *E. stiedae*.

Testes.—Atrophy of these organs was usually present.

Amyloid was not found in these animals and no other characteristic lesions were noticed. The skin appeared normal.

Rabbits suffering from " runt disease " (Group IV).

All the animals in this group failed to gain weight at the normal rate. The severity of the disease varied greatly, so that whereas a few of the baby rabbits died within 15 days of birth following an " acute " attack, others suffered from a " chronic " attack causing great retardation of growth and death 50–70 days after birth. The mean survival time of those dying naturally was 31·93 $\pm$ 12·79 days.

Three runts were skin grafted from the spleen donor and proved to be fully tolerant for as long as they lived.

Bone marrow.—Of the 14 animals that died from " runt disease ", 3 showed an aplastic bone marrow with loss of most of the haemopoietic cells (Fig. 11 and 12) and 5 a femoral marrow greatly depleted of cells (Fig. 13). These changes were accompanied by clinical evidence of immune haemolysis of the animals' own red cells and a steep fall in the leucocyte and platelet counts (Porter, 1960*c*). The other 6 animals showed in 2 cases a bone marrow of normal cellularity and in 4 a granulocytic hyperplasia. Three of the rabbits with normal or hyperplastic bone marrow were males and smears showed occasional heterophils with " drumsticks ", and similar female cells were found in the peripheral blood, showing that some at least of the bone marrow cells were derived from the spleen donor.

Of the 6 animals killed at various times after birth, the 2 examined at one week both showed a bone marrow of normal cellularity in which female cells could not be found ; the 2 rabbits killed at 2 weeks showed loss of cellularity of the marrow ; and the marrow of 1 animal examined at 3 weeks was aplastic, whilst the other showed masses of donor type heterophils and their precursors.

Spleen.—In all 14 animals with " runt disease " that died naturally, the spleen appeared normal size in 8, and small in the remainder. Microscopically there was great diminution in the number of lymphoid cells in the splenic nodules and white pulp generally (Fig. 14 and 15). At best only a small halo of lymphocytes and mature plasma cells was left around the splenic arterioles ; and in 6 animals the Malpighian bodies had been completely replaced by immature or mature collagen (Fig. 16). The red pulp was congested in many, and erythrophagocytosis was prominent in all spleens. Extramedullary haemopoiesis, although present in some, was never very marked.

One of the rabbits killed a week after birth showed apparently normal lymphoid nodules in the spleen, but the other animal had an enlarged spleen with

many transitional cells and some immature plasma cells in the white pulp. Cell suspensions from these spleens were examined by the method of Riis (1957), and some mononuclears with female sex chromatin found.

EXPLANATION OF PLATES

FIG. 1.—Bone marrow from a rabbit killed 14 days after the onset of " secondary disease ". The marrow remains well repopulated, but there is loss of fat. Smears showed female cells. H. and E. × 110.

FIG. 2.—Spleen from a rabbit killed 7 days after the onset of " secondary disease ". The lymphoid nodule is well repopulated with transitional cells, immature plasma cells and a few lymphocytes. H. and E. × 110.

FIG. 3.—Spleen from a rabbit killed 14 days after the onset of " secondary disease ". Many of the cells in the lymphoid nodule are necrotic. H. and E. × 50.

FIG. 4.—Spleen from a rabbit killed 14 days after the onset of " secondary disease ". The lymphoid nodule is depleted of cells and partly replaced by masses of smudgy fibrinoid material. H. and E. × 50.

FIG. 5.—Spleen from a rabbit killed 21 days after the onset of " secondary disease ". The white pulp has been replaced by immature collagen and numerous foreign body giant cells. H. & E. × 50.

FIG. 6.—Spleen from a rabbit killed 21 days after the onset of " secondary disease ". The lymphoid tissue has disappeared and its place taken by fibrous tissue and a few scattered plasma cells. H. and E. × 50.

FIG. 7.—Inguinal lymph node removed at biopsy from a rabbit 14 days after irradiation and marrow treatment. There is incomplete repopulation with lymphocytes. Female sex chromatin was demonstrated in some of the cells from this node. H. and E. × 50.

FIG. 8.—Inguinal lymph node removed at post mortem from a rabbit killed 14 days after the onset of " secondary disease ". Note that many of the lymphoid cells have undergone necrosis. A biopsy of a node from the other groin of this animal, before the onset of " secondary disease ", is shown in Fig. 7. H. and E. × 50.

FIG. 9.—Higher magnification of part of a mesenteric lymph node from a rabbit killed 14 days after the onset of " secondary disease ". Mature plasma cells predominate amongst the surviving cells. H. and E. × 400.

FIG. 10.—Mesenteric lymph node from a rabbit killed 21 days after the onset of " secondary disease ". Lymphocytes have disappeared, only reticular cells and a sprinkling of mature plasma cells remain. H. and E. × 50.

FIG. 11.—Femoral bone marrow from a normal baby rabbit 30 days old for comparison with Fig. 12. H. and E. × 110.

Fig. 12.—Bone marrow from a baby rabbit that died from " runt disease " when 30 days old. There is almost complete aplasia of haemopoietic cells. Compare with Fig. 11 which shows the normal appearance in a rabbit of this age. H. and E. × 110.

FIG. 13.—Marrow from a rabbit that died from " runt disease " when 40 days old. There is great depletion of haemopoietic cells when compared with the marrow shown in Fig. 11. H. and E. × 110.

FIG. 14.—Spleen from a normal baby rabbit 21 days old for comparison with Fig. 15. H. and E. × 50.

FIG. 15.—Spleen from a rabbit with " runt disease ", killed at 21 days. The lymphoid nodule contains very few lymphocytes. Compare with Fig. 14 which shows the normal appearence in a rabbit of this age. H. and E. × 50.

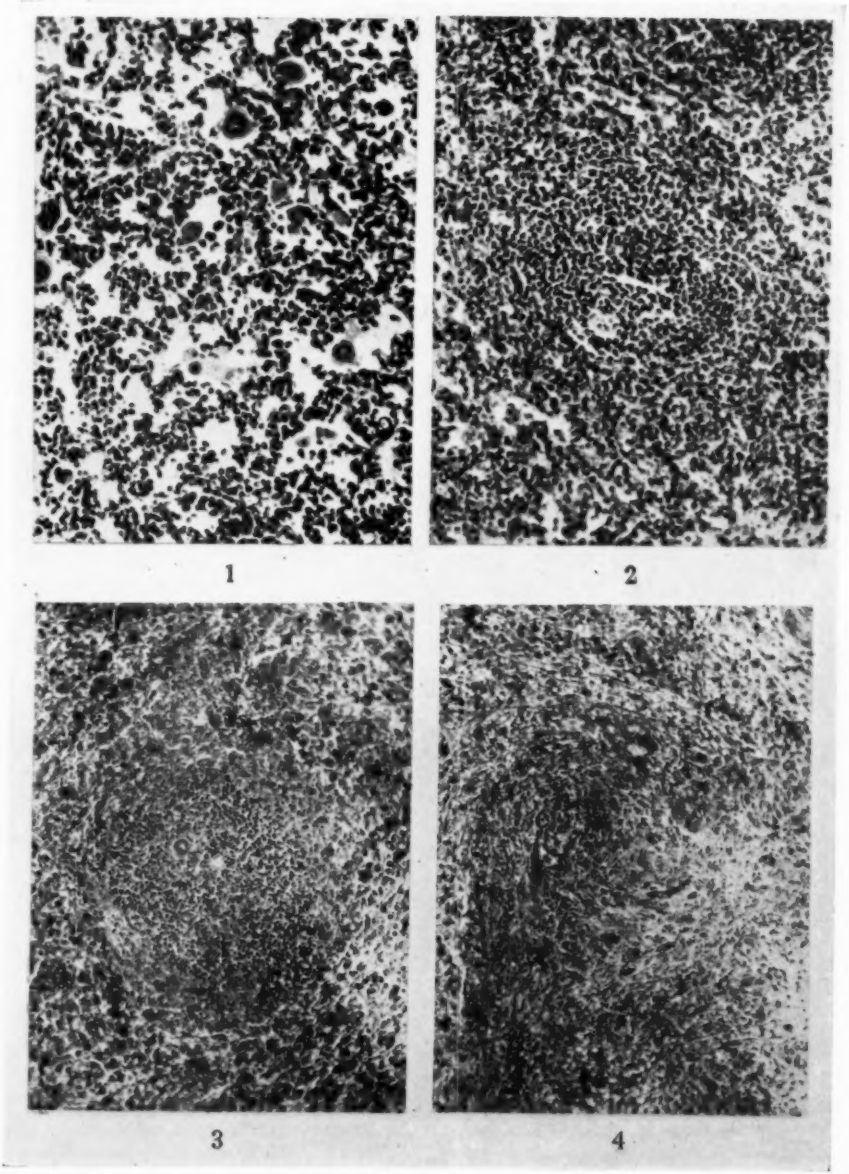
FIG. 16.—Spleen from a rabbit that died from " runt disease " when 70 days old. There is loss of lymphoid tissue and replacement by fibrous tissue. H. and E. × 140.

FIG. 17.—Mesenteric lymph node from a normal baby rabbit 20 days old for comparison with Fig. 18. H. and E. × 50.

FIG. 18.—Mesenteric lymph node from a rabbit that died from " runt disease " when 20 days old. Lymphocytes have disappeared, but reticular cells and mature plasma cells remain. Compare with Fig. 17 which shows the normal appearance in a rabbit of this age. H. and E. × 50.

FIG. 19.—Appendix from a normal baby rabbit 21 days old for comparison with Fig. 20. H. and E. × 50.

FIG. 20.—Appendix from a rabbit with " runt disease " killed at 21 days. There is almost complete loss of lymphoid tissue when compared with Fig. 19 which shows the normal for this age. H. and E. × 50.



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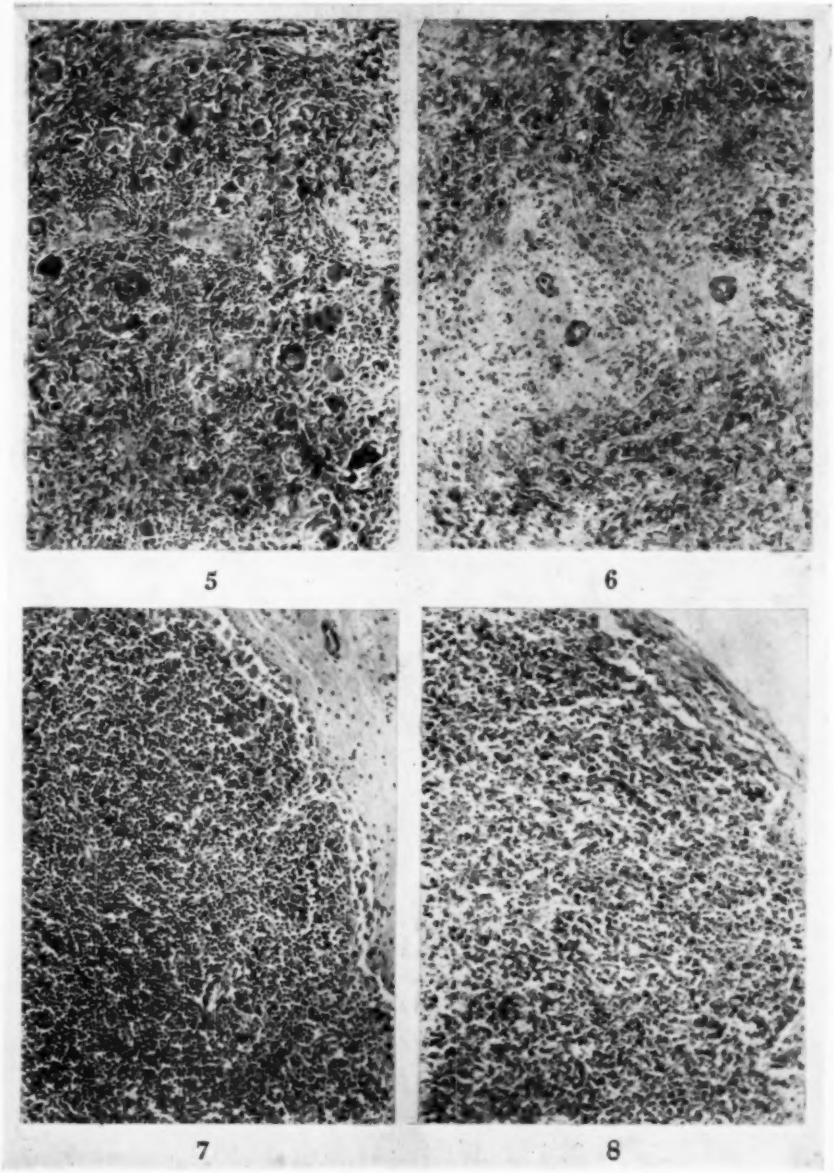

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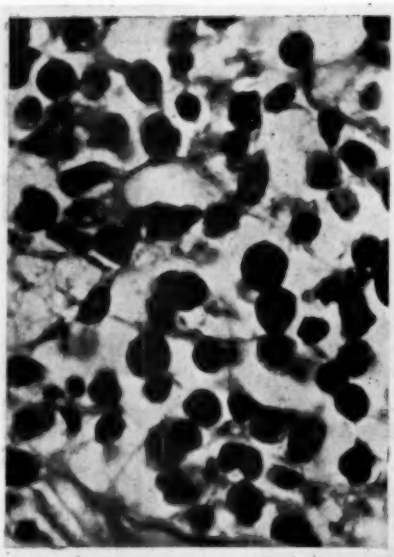

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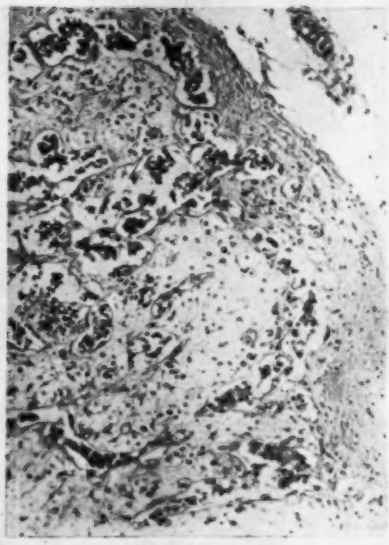

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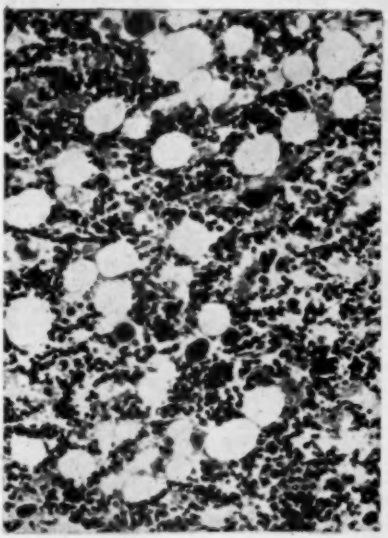

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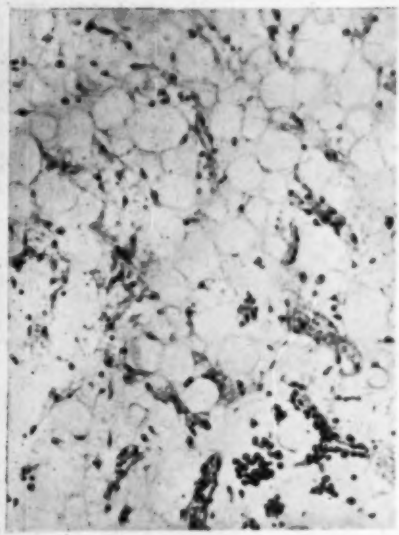

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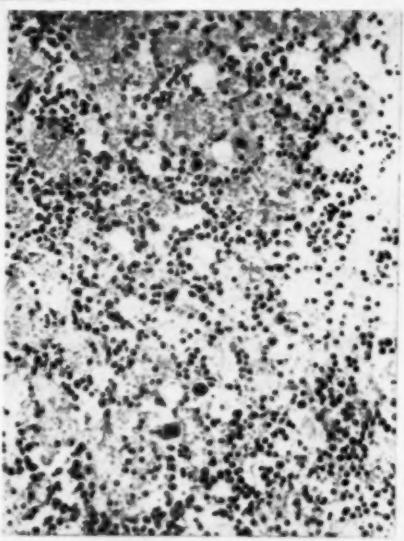

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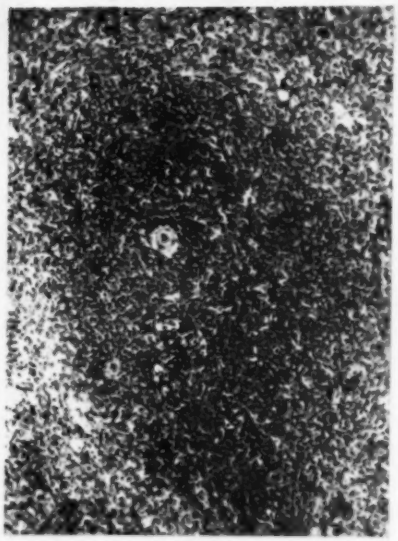

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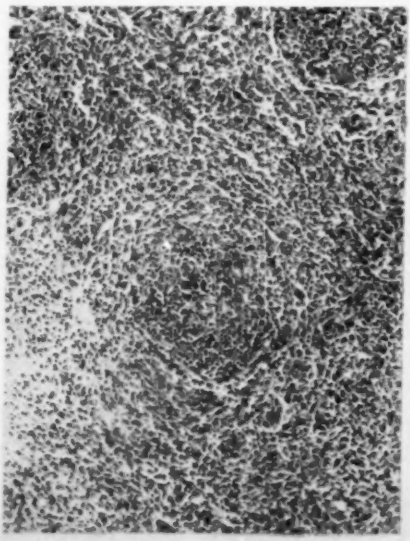

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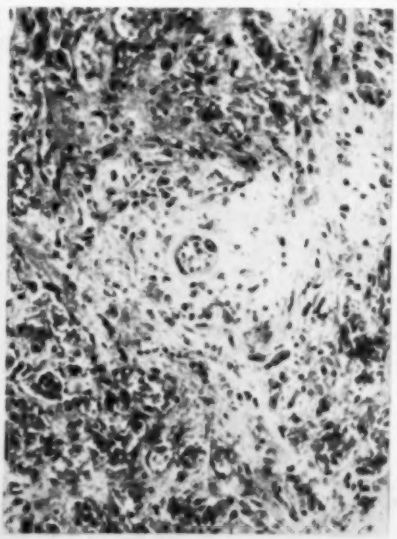

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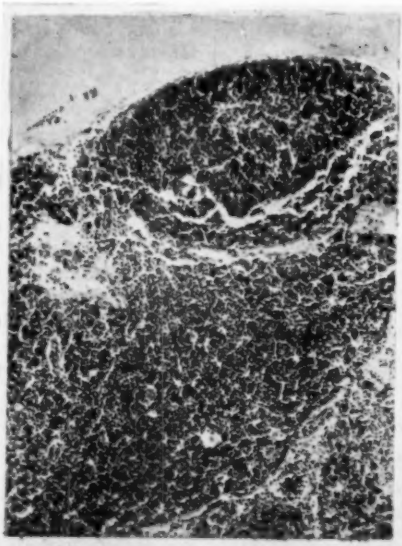

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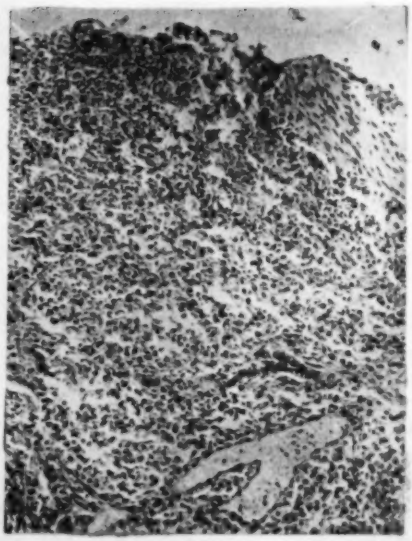

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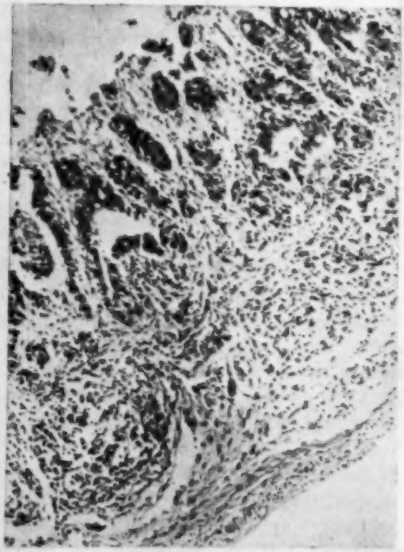

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The spleens of the animals killed at 2 weeks were normal size, but both microscopically showed proliferation of transitional and immature plasma cells in the white pulp with few lymphocytes and 1 rabbit showed in addition small patches of fibrinoid necrosis in the lymphoid nodules.

The 2 animals killed at 3 weeks both had small spleens with great reduction in lymphoid content of the white pulp and in 1 of the rabbits the splenic nodules had been replaced by collagen.

Lymphoid tissue elsewhere.—All the animals that died from " runt disease " showed, compared with controls of the same ages, shrunken lymph nodes containing very few lymphocytes (Fig. 17 and 18). This loss of lymphocytes was also a striking feature of the intestinal lymphoid tissue, e.g., Peyer's patches, and was most obvious in the appendix (Fig. 19 and 20). The thymus was however, an exception, for although there was undoubtedly some decrease in lymphocytes, this was nowhere so obvious as in lymphoid tissue elsewhere.

In the animals killed at intervals after birth, primitive cells, including transitional and immature plasma cells, were numerous in the lymph nodes at 1 and 2 weeks, but by 3 weeks the nodes were atrophic. Lymphocytes were at all times scarce.

Respiratory system.—The lungs of 12 of the 14 animals that died from " runt disease " showed bronchopneumonic lesions at post mortem. In the 8 animals with an aplastic or hypoplastic bone marrow this was a necrotic and haemorrhagic non-purulent infection, whilst in the remainder it took the form of a purulent peri-bronchiolar consolidation.

Gastro-intestinal tract.—Apart from an ulcerative enteritis in 3 animals, no other specific lesions were seen.

Liver.—In the livers of 6 of the 14 animals that died, and in the liver of 1 of the animals killed at 3 weeks, there were small focal areas of necrosis. These lesions were identical with those seen in the irradiated rabbits suffering from " secondary disease ". Evidence of coccidiosis was sought but not found.

No renal or vascular lesions were observed in any of the animals. The skin and other organs appeared normal. Amyloid was not found in any of the rabbits.

DISCUSSION

As the present study shows the pathological changes in " runt disease " are very like those seen in " secondary disease ". In both cases the animals become progressively more sick, waste and often develop diarrhoea. Death from infection usually occurs some 2–6 weeks after the onset of the process. In both instances by using sex chromatin as a biological marker it has been shown that the foreign cells injected, or their descendants, persist and are present in the lymph nodes and spleen, i.e., these animals are cellular chimaeras. At first free proliferation rapidly repopulates the host's lymphoid system with poorly differentiated pyronin-positive donor cells resembling the transitional and immature plasma cells described by Fagraeus (1948). After this initial increase there is a generalised regression with atrophy of the new lymphoid tissue and its gradual replacement by collagen and scattered collections of mature plasma cells. Focal liver necroses, which have been discussed previously (Porter, 1960*b*), are also a feature of both syndromes.

When the haemopoietic tissues are considered, however, certain differences are apparent between " runt disease " and " secondary disease ". In " secondary disease " there is rapid repopulation of the aplastic bone marrow, and even when the rabbit is dying this restored marrow always remains cellular and generally shows an extensive granulocytic hyperplasia. Use of the " drumstick " marker shows that this colonisation is from proliferation of donor cells, and this is confirmed by finding female type heterophils in the peripheral blood.

In " runt disease ", on the contrary, the bone marrow is frequently destroyed and the animal develops a severe anaemia. Only in a few runts is this destruction of host bone marrow accompanied by progressive replacement with haemopoietic cells of donor origin.

This difference is not difficult to understand when one remembers that in producing radiation-chimaeras a very large dose of haemopoietic cells and their precursors is given intravenously to an animal whose own bone marrow has been destroyed by X-rays ; whereas in producing runts spleen suspension containing relatively few such haemopoietic cells is given intraperitoneally to an animal whose own bone marrow is intact.

In both instances an immune haemolysis of host red cells is seen, accompanied by a raised indirect serum bilirubin, erythrophagocytosis and a positive Coomb's test. Also in both, as the disease progresses, the peripheral blood lymphocyte count fails steadily (Porter, 1960*c*).

These observations underline the essential similarity between " secondary disease " and " runt disease " in the rabbit. In both it seems the host is immunologically defenceless : in " secondary disease " because of irradiation damage, in " runt disease " because the immune system is insufficiently mature. The injected foreign cells proliferate, invade, and repopulate the lymphoid system of the host. Indirect evidence then suggests that they proceed to attack the host cells, but how, and whether this involves antibody, is not known. Further, why histological evidence of such an attack should be confined to haemopoietic, lymphoid and possibly hepatic tissue, is equally obscure. As the postulated assault continues, destruction of the restored lymphoid tissue also gradually occurs. At present, no explanation of this secondary loss of donor cells is entirely satisfactory. Kaplan and Rosston (1959) suggest that the foreign cells probably die in the course of killing host target cells : a process which they envisage may necessitate direct contact between each donor cell and a very few target cells. It may even be that the excessive amount of host antigenic material coming to the foreign lymphoid tissue simply overwhelms and exhausts it. However, it is important to be clear that no experimental precedent for such " exhaustion " exists. Whatever the exact mechanism, it does seem that death of the chimaera is the outcome of destruction of the antibody-producing cells of both host and donor.

As might be anticipated, giving irradiated rabbits homologous lymph node suspension as well as bone marrow leads to rapid repopulation of the host's spleen and lymph nodes with foreign cells and the onset of an " accelerated " form of " secondary disease " (Porter, 1960*b*).

Conversely, the incidence of " secondary disease " can be greatly reduced by using foetal haemopoietic cells, which are known to be immunologically immature, instead of adult bone marrow to produce the radiation-chimaeras (Uphoff, 1958 ; Porter, 1959.)

Other examples of graft-against-host reactions have recently been recognised

and although the details of each syndrome vary according to the circumstances of the experiment, the end result is always wasting and lymphoid atrophy.

Thus, if splenic cells from parental strain donors are injected into F1 hybrid mice a wasting disease results which closely resembles " secondary disease " (Trentin, 1958). In this instance the foreign cells are not rejected because the F1 hybrid mice possess in their tissues all the antigens of both parent strains. The injected lymphoid cells therefore proliferate and attack the host producing histological changes very like those described in the present paper (Nowell and Cole, 1959).

Similarly, many rat pairs placed in parabiosis with a cross-circulation develop " parabiosis intoxication ", in which one animal remains well while the other rapidly wastes and his lymphoid tissue atrophies. When such homologous rats are separated after 5 days in parabiotic union a previously exchanged skin homograft may persist in an animal which is dying with general lymphoid atrophy and severe anaemia (Nakič and Silobrčič, 1958).

It thus seems that any transplantation of immunologically competent cells into an animal whose immune defences are for some reason paralysed, is potentially hazardous and may, under conditions favourable to the invading cells, produce a lethal wasting syndrome.

SUMMARY

Secondary irradiation disease and " runt disease " in rabbits are described and compared.

Outstanding features of both are progressive wasting and diarrhoea, early splenomegaly and enlargement of lymph nodes, followed later by shrinkage of these organs and focal liver necroses. Overwhelming infection is usually the immediate cause of death.

With the help of sex chromatin and " drumstick " markers, it is shown that in both instances the animals are cellular chimaeras. There is early invasion and repopulation of host lymphoid tissues by proliferating donor-type transitional cells and immature plasma cells. Later, these cells undergo necrosis and this is sometimes associated with fibrinoid changes and foreign body giant cells. The end result is extreme lymphoid atrophy with fibrosis.

The only histological difference between the two conditions is that in " secondary disease " the aplastic bone marrow is rapidly colonised with donor cells and is still highly cellular when the animal dies, whereas in " runt disease " the host's bone marrow is frequently destroyed and less often is there repopulation with donor-type haemopoietic cells.

The similarity between these syndromes and those known as " parabiosis intoxication " and F1 hybrid " wasting disease " is noted and the conclusion reached that they are all graft-against-host reactions.

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REFERENCES

BILLINGHAM, R. E. AND BRENT, L.—(1957) *Transplant. Bull.*, **4**, 67.
COLE, L. J. AND ELLIS, M. E.—(1958) *Science*, **128**, 32.
CONGDON, C. C. AND URSO, I. S.—(1957) *Amer. J. Path.*, **33**, 749.
DEMPSTER, W. J.—(1953) *Brit. J. Surg.*, **40**, 447.
FAGRAEUS, A. (1948) *Acta med. scand.*, Suppl. 204.
GORER, P. A. AND BOYSE, E. A.—(1959) *Immunology*, **2**, 182.
KAPLAN, H. S. AND ROSSTON, B. H.—(1959) *Stanf. med. Bull.*, **17**, 77.
MEDAWAR, P. B.—(1958) *Proc. Roy. Soc.* B., **148**, 145.
NAKIČ, B. AND SILOBRČIČ, V.—(1958) *Nature, Lond.*, **182**, 264.
NOWELL, P. C. AND COLE, L. J.—(1959) *Transplant. Bull.*, **6**, 435.
PORTER, K. A.—(1957*a*) *Ibid.*, **4**, 129.—(1957*b*) *Brit. J. exp. Path.*, **38**, 401.—(1959) *Ibid.*, **40**, 273.—(1960*a*) *Nature, Lond.* **185**, 789.—(1960*b*) *Clin. Radiol.*, **11**, 22.—(1960*c*) *Ann. N.Y. Acad. Sci.* (in press).—(1960*d*) *Brit. J. exp. Path.* **41**, 72.
Idem AND MURRAY, J. E.—(1958) *J. nat. Cancer Inst.*, **20**, 189.
RIIS, P.—(1957) *Nature, Lond.*, **179**, 785.
SIMONSEN, M.—(1953) *Acta path. microbiol. scand.*, **32**, 36.—(1957) *Ibid.*, **40**, 480.
STARK, R. B., BROWNLEE, H. AND GRUNWALD, R. P.—(1958) *Ann. N.Y. Acad. Sci.*, **73**, 772.
TRENTIN, J. J.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **92**, 688.—(1958) *Ann. N.Y. Acad. Sci.*, **73**, 799.
UPHOFF, D. E.—(1958) *J. nat. Cancer Inst.*, **20**, 625.

KIDNEY CARCINOMAS OF THE FOWL INDUCED BY THE MH2 RETICULOENDOTHELIOMA VIRUS

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IT has been shown that the ES4 virus causing erythroleukaemia of fowls would also cause renal carcinomas in very young chicks (Carr, 1956). It therefore seemed opportune to re-examine the kidney tumours caused by the MH2 virus that were described by Foulds (1934*a*, 1934*b*). Though he concluded that " there is no reason to implicate the filterable agents in their production " he regarded some as atypical metastases with an epithelial element, saying of one that it " scarcely permitted any other name than adenoma (or adenocarcinoma) ", a remark which the illustration certainly appeared to confirm completely. Day-old animals were sometimes used in his experiments, but no correlation of the " adenomas " with the use of young hosts was mentioned, and his attempts at transplantation of the adenomatous structures failed. He stated that the opinions on their nature and production could only be regarded as provisional.

MATERIALS

The virus used in this work was revived from a vial of freeze-dried tumour that had been prepared by Dr. P. R. Peacock at Glasgow over 18 years previously. The fowls were all from the Centre's flock of Brown Leghorns.

METHODS

Virus preparations consisted of either a 10 per cent extract of macerated tumour tissue in water, clarified by centrifugation at about 3000 *g* for 10 minutes, or such an extract further purified by separation of the virus by high-speed centrifugation and enzyme digestion (Bather, 1953). Cell grafts were made from a 20 per cent suspension of tumour cells in saline which had been allowed to settle for about 10 minutes. All injections were of 0·2 ml. injected through a very fine (intradermal) needle.

RESULTS

Most of the kidney tumours were obtained in a similar way to that described for the erythroleukaemia virus ; decimal dilutions of a virus preparation were injected intramuscularly into the legs of one or more groups of chicks of known age, and the animals killed and examined as soon as the tumour began to interfere with their well-being. To these were added a few carcinomas encountered in birds injected with an arbitrary dose of material in connexion with other experiments, but this was found to be a very inefficient way of inducing the kidney tumours, as the time interval between inoculation and death was usually too short (see below).

Such investigations emphasised a very marked difference between the reactions of birds of different ages to the primary inoculation, which will be first described.

Growth of the primary tumour

There was a very obvious difference in the growth of the MH2 tumour, whether originating from cells or virus, depending upon whether the inoculation was made into chicks less than one week old or into older birds. The usual slow progression of the tumour as seen in birds of six weeks old or more, which seldom fills the muscle in less than 4–5 weeks and often regresses, changed to a rapid proliferation, which frequently inconvenienced the animal at about 14 days. Even when inoculated at the age of about nine days, tumour growth was slower and they frequently regressed, so that titration by the methods of limiting dilution was impractical at this age, while survival of younger chicks for the 26 days or so that were found to be needed to produce kidney tumours in the case of the ES4 erythroleukaemia virus was likely to be rare. This difference was accentuated by the variation in the metastatic spread of the tumours described in the next section.

Dissemination of the tumour

The spread of the tumour cells in the body of the host was also sharply different in the young chicks. In birds over 10 days of age metastases were of moderate frequency, usually seen as a few discrete white or yellow sharply-circumscribed nodules in the liver, and less frequently in the spleen, proventriculus and kidney.

In contrast the very young chicks often suffered a massive dissemination of tumour cells which produced a striking resemblance to leukaemia. The liver and spleen were often pale and grossly enlarged, due to a diffuse infiltration of malignant cells whose mass sometimes was obviously greater than the primary. The bone-marrow was similarly involved, and primitive cells were present in the circulating blood. The general picture would certainly have led to the diagnosis of leukaemia from the post-mortem appearance alone.

Intermediate stages, with massive diffuse metastases into the viscera were also often encountered.

Table I gives a summary of such an experiment using a rather heavy dose of virus, which, despite the difference in age of the chicks of only 7 days, shows the considerable difference in the reaction to the virus.

TABLE I.—*Showing difference in Reaction to Virus at Two Ages*

Age (days)	Number	Primary tumour	Leukaemoid	Survival (days)
1	8	8	6	11–21
8	8	4	0	19–28

The experiment was terminated and survivors killed at 28 days.

This association of a leukaemia-like condition with MH2 was also noticed by Foulds (1934) who reported that a "minority of birds inoculated with MH2 die of a disease which has some features in common with leukosis". The present finding, that this is confined to the very young hosts, was not mentioned by him. The relation of this to leukosis is referred to again in the discussion ; it is mentioned

here since, as will be realised, it contributed to the difficulties in obtaining the kidney tumours.

Kidney growths

(*a*) *Gross appearance.*—In experiments with older chicks, kidney involvement was rather rare, in contrast to the youngest animals, which showed obvious growths more often than not. These varied from a single lump which might involve the whole of one kidney to over a dozen of assorted sizes in each organ. Some, usually the larger ones, were solid and deep-lying, while others were superficial and occasionally cystic. As Foulds noted, most of these were found in birds surviving three weeks or more.

(*b*) *Microscopic.*—Histologically, these kidney tumours were of two distinct kinds. One class was obviously a purely metastatic growth of ordinary MH2 cells, which started as an intertubular mass separating the tubules before their invasion and destruction occured, as described by Murray and Begg (1930), Foulds (1934) and others. Only this type was found in birds injected at an older age, and in the younger animals which died early.

Contrasting with this was a kidney tumour confined to birds injected at the age of 10 days or less—usually the groups inoculated at a few days—and surviving for 26 days or more; these limits being very similar to those found for the erythroleukaemia virus. These were, as Foulds remarked, frankly carcinomatous growths of columnar eipthelium, and there seems no reason to avoid calling them anything other than kidney carcinomas.

The small and early forms are of the type shown in Fig. 1. Ingrowths from a simple cyst yield a papillomatous adenocarcinoma, whose central space is later filled by the cancerous material to produce a picture of the type shown in Fig. 2. Here the epithelial cells are forming multiple alveoli, which are sometimes filled by necrotic material. These adenomatous or alveolar structures are separated in places by a connective tissue stroma. Such growths may become very large, replacing almost the whole of the affected kidney by a bulky tumour. Invasion of adjacent viscera does not occur. The tumours have all had this rather simple structure, and they do not show any of the complexities characteristic of embryonal nephromas.

The epithelial cells usually contain a rather large and deeply-staining nucleolus (Fig. 3), characteristic also of the sarcomatous form of the MH2 tumours. These epithelial cells are extremely basophilic, much more so than the ordinary MH2 tumours. Fig. 1 shows an area of ordinary MH2 sarcoma metastasis adjacent to a carcinoma whose very deeply stained cytoplasm makes the differentiation easy.

Small carcinomas have usually been found at the periphery of the organ and like the erythroleukaemia ones seem to have their origin in the nephrogenic areas still to be found in this region.

The results of a typical experiment are given in Table II. This was performed on 3-day old birds, and the yield of three carcinomas out of 18 animals is rather better than the average.

Transplantation

The conditions under which carcinomas were induced having been established, attempts were made to transplant them. Kidney growths occurring in suitable birds were selected, though of course it could not be certain beforehand that they

TABLE II.—*Showing the Number of Carcinomas Induced in a Typical Experiment*

Virus dose (equivalent tumour weight)	Survival (days)	Metastases other than kidney	Kidney involvement	Carcinomas
$0{\cdot}2 \times 10^{-3}$	33	+	++	+
	14	++	−	..
$0{\cdot}2 \times 10^{-4}$	32	+	−	..
	32	++	++	+
	33	+	++	+
	15	+	−	..
$0{\cdot}2 \times 10^{-5}$	33	−	−	..
	19	−	−	..
	19	++	++	−
	33	−	−	..
$0{\cdot}2 \times 10^{-6}$	19	+	++	−
	33	−	−	..
	32	+	−	..
	*	..	..	..
$0{\cdot}2 \times 10^{-7}$	33	−	−	..
	*	..	..	..
	*	..	..	..
	*	..	..	..

* Means no primary tumour induced.
The experiment was terminated and all animals killed at 33 days.

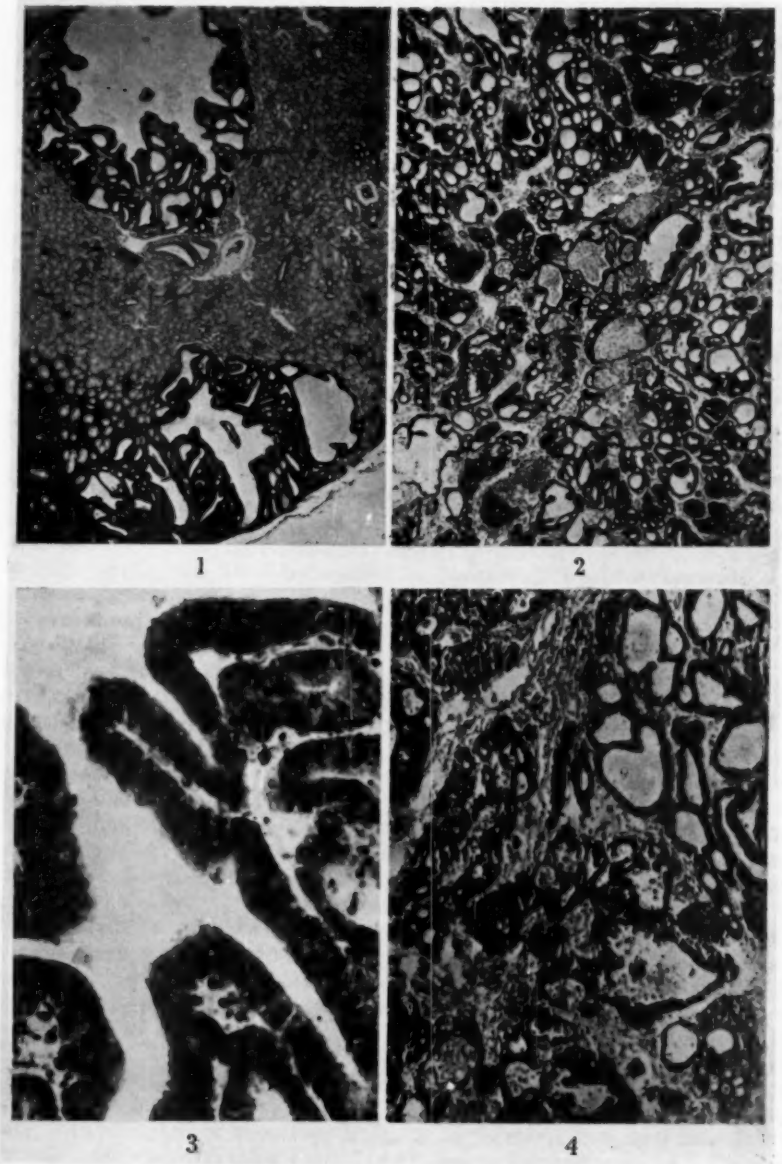
were not merely metastatic sarcomas. One half of the tumour was used to prepare histological sections, and the other half provided material for grafting into groups of 4–12 birds. In practice, about half of those selected proved to be carcinomatous. The results of these experiments can be summarised as follows :

Carcinomas were almost always transmitted to nearly all the hosts aged 1–18 days, but the same material would invariably fail to produce anything but a sarcoma in birds aged 30 days or more. In very young chicks the transplant proliferated rapidly, and could incapacitate the chick in about 15 days. The cut surface of successfully-transplanted carcinomas was characteristic ; instead of the plain waxy surface of the sarcomatous MH2, much of it showed a complex pattern of whorls. Section showed areas of ordinary MH2 sarcoma mingled with extensive carcinomatous areas of the type shown in Fig. 4. The proportions varied, but it was possible to get over 10 g. of transplant, the majority of which resembled this picture. Other tumours yielded only a small central area of carcinoma, amounting to less than a gram in all.

As Fig. 4 indicates, the carcinomatous parts of these grafts did not usually look as healthy as did the primary renal tumour, and gave the impression of reverting to the sarcomatous structure. It was therefore not surprising that attempts to transplant these tumours for a second generation gave no convincing

EXPLANATION OF PLATE

FIG. 1.—Showing early cystic carcinomas. Surface of kidney bottom right. Sarcomatous metastases bottom left.
FIG. 2.—Portion of large carcinoma.
FIG. 3.—Carcinoma. higher magnification ; note large nucleoli.
FIG. 4.—Transplanted carcinoma.



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Carr.

evidence for the further proliferation of the carcinomatous portion, though it is not yet clear whether this is due to reversion of the cells, death, or simple overgrowth by the sarcomatous portion.

Metastases in birds carrying carcinomatous transplants were always sarcomatous in type.

DISCUSSION

These tumours are clearly analogous to the papillary adenocarcinomas induced by the ES4 erythroleukaemia virus (Carr, 1956) which are undoubtedly of renal origin, and there can be no valid reason for not regarding and naming them as such. Determination of their virus content was not made. Because of the certain contamination by infective blood, and possible contamination by sarcomatous areas as in Fig. 1, these could not be reliable and might be misleading if any importance was attributed to the results. Foulds (1934) hesitated to consider them as true carcinomas and referred to the work of Lauterberg (1919) on kidney metastases in humans to explain the alveolar patterns in such tumours. The frankly carcinomatous growths do not, in fact, particularly resemble Lauterberg's description of such metastases, where the kidney tubules were replaced by carcinoma cells, the glomeruli remaining intact, to give an architecture still recognisable as kidney but whose tubules were made from cells of the primary growth. The MH2 kidney carcinomas are formed by cells that are cytologically distinct from the MH2 sarcoma cell of the primary tumour, as Fig. 1 and Fig. 3 show, and cannot be mere secondaries derived from it.

Definite proof of this contention is provided by the transplantation experiments, where a suspension of cells sufficiently disaggregated to pass a fine intradermal needle proliferated in muscle to give large areas recreating the original pattern.

These transplantation experiments, if they thus aid in solving one problem, pose a new one, that of the age limitation for successful transmission as a carcinoma. Such a limitation for carcinoma, namely growth as a sarcoma in older animals, but as a carcinoma in young ones, is unknown in other aspects of cancer research. However, with this information gained with the MH2 virus, it has been found that the ES4 erythroleukaemia virus carcinomas, previously regarded as non-transmissible (Carr, 1956), can in fact be similarly transplanted by cell grafts if very young hosts are employed (Carr, unpublished). A similar factor may have been operating when Duran-Reynals (1946) only obtained sarcomas from fowl embryonal nephromas. If this change can be technically regarded as an irreversible carcinoma to sarcoma transformation of the graft it is not at first sight analogous with what is usually understood by this in the case of mammalian tumours, though this may be in part a consequence of its great speed. Comparable information of the age of the host in relation to this change does not seem to be available for mammals.

The study of this aspect of the fowl carcinomas is rendered very difficult because in each case virus liberated from the graft of the carcinoma will form a sarcoma at the inoculation site, and it appears that this is the faster-growing element. In the case of the older fowls, it is therefore not clear whether the sarcoma was derived from the carcinoma cells, or was induced by virus. Certainly, the grafts in young chicks appeared to be reverting by cellular change (Fig. 4) but even here this might be a reflection of the competition by the sarcomatous

elements induced on inoculation as these carcinomas are disintegrating in a host younger than the original carrier.

The correlation of age of the host and reaction to MH2 virus is indeed complex, as the work of Dhaliwal (1959) showed in the embryonic system. His work clearly proved that infection of the embryo cells by circulating virus was possible without the necessity for injury. As in this work, it is very likely that the leukaemia-like reaction of the young host is a true one, caused by infection of the haemopoietic cells by circulating virus. It also seems likely that, corresponding to the carcinomas, this is limited to a brief span of days, and then reverts to a sarcomatous process. This equally makes definite proof of a true leukaemia difficult, and is in part dependent upon the definition of leukaemia that is accepted. At present, the condition is perhaps better referred to by a non-committal term like " leukaemoid ".

If some of these aspects appear ill-defined and uncertain, this is a property they share with the fowl leucosis complex to which they belong. The relationship of the "leukaemoid" reaction of MH2 birds to leucosis was early recognised by Foulds (1934) and is quoted above. The fowl leucosis complex has been held to include lymphoid, myeloid and erythroleukaemias, sarcomas, osteopetrosis, osteosarcomas, fowl paralysis (neurolymphomatosis) and ocular lymphomatosis, to which kidney carcinomas have been added by these investigations on the ES4 and MH2 renal tumours. For a recent review of this complex see Campbell (1956). Few of these diseases have had any detailed investigation of the potentialities of their causative virus(es). On the other hand the relatively uncomplicated Rous 1 virus, which causes neither kidney carcinomas nor leukaemias in young chicks (Carr, 1959) was shown by Duran-Reynals (1947) to produce many new varieties of cancer when modified by heterotransplantation. The complexities of the work are, most probably, greater than has been appreciated, rather than less.

SUMMARY

The MH2 virus of reticuloendothelioma will also induce kidney carcinomas in very young chicks. These lesions are only found when the host survives for several weeks, and seem analogous to those induced by the ES4 virus. They can be transplanted for one passage to young hosts, but in older ones only sarcomas result. The occurrence of a leukaemia-like condition with MH2 virus has been confirmed. It is pointed out that it is also mainly a reaction of the young host whose response to the virus changes rapidly shortly after hatching.

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REFERENCES

Bather, R.—(1953) *Brit. J. Cancer*, **7**, 492.
Campbell, J. G.—(1956) *Vet. Rec.*, **68**, 527.
Carr, J. G.—(1956) *Brit. J. Cancer*, **10**, 379.—(1959) *Virology*, **8**, 269.
Dhaliwal, S. S.—(1959) *Brit. J. Cancer*, **13**, 685.
Duran-Reynals, F.—(1946) *Cancer Res.*, **6**, 545.—(1947) *Ibid.*, **7**, 99.
Foulds, L.—(1934*a*) *Sci. Rep. Cancer Res. Fd, Lond.*, **11**, 1.—(1934*b*) *Ibid.*, **11**, 15.
Lauterberg, A.—(1919) *Z. Krebsforsch.*, **16**, 442.
Murray, J. A. and Begg, A. M.—(1930) *Sci. Rep. Cancer Res. Fd, Lond.*, **9**, 1.

STUDIES ON MOUSE LEUKAEMIA. LEUKAEMOGENESIS BY CELL-FREE FILTRATES INOCULATED IN NEWBORN AND ADULT MICE

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FILTERED extracts, usually from neoplastic tissues, can induce leukaemias in newborn or adult mice of certain strains (Gross, 1957*a* ; Graffi, 1957 ; Friend, 1957 ; Schwartz and Schoolman, 1959). Gross has shown that such extracts from tissues of high-leukaemic strain mice with spontaneous lymphocytic leukaemias, when injected at birth into mice of low-leukaemic strains, will ultimately produce leukaemia in about a third of the recipient mice (Gross, 1958*b*). The resulting leukaemias were transplantable by cell graft in most cases to adult mice of the recipient line but only rarely to the donor strain in which the original leukaemia occurred.

Only newborn mice less than 16 hours after birth were found initially to be susceptible to the leukaemogenic activity of the extracts. When, however, the extracts were passed serially through several generations of susceptible mice, their potency was increased and young adult mice could be successfully inoculated (Gross, 1958*c*). Marked inter- and intra-strain sensitivity has been found in the response of the mice to inoculation of the extracts. In the C3H strain, for instance, the C3Hf/Gs subline is the most susceptible (Woolley and Small, 1957) while the C3Hf/An subline is hardly sensitive (Gross, 1955*a*). The necessity of using newborn mice and the marked differences in strain and substrain susceptibility have suggested the possibility that the disease only develops in mice that can acquire immune tolerance to the leukaemic agent (Harris, 1958 ; Barnes *et al.*, 1959).

A series of investigations has been commenced in this laboratory in an attempt to elucidate the mechanism of leukaemogenesis by cell-free extracts of leukaemic tissues. The present paper reports the results obtained in four different strains of mice inoculated as newborn or as adults with filtered extracts. A further paper is concerned with the role of the thymus in leukaemogenesis by cell-free filtrates of leukaemic tissues (Miller, 1960). A preliminary account of these studies has already been given (Miller, 1959).

MATERIALS AND METHODS

Mice.—Experiments were carried out on mice of four different inbred strains (Table I). Inbreeding in mice of all four strains has been maintained by strict sib-matings in our laboratory at Pollards Wood since the time of their acquisition.

Filtrates.—Cell-free filtrates were prepared from freshly obtained tissues by a method closely following that of Gross (1958*b*). The tissues were homogenized in

TABLE I.—*Source and Date of Acquisition of Inbred Strains*

Strain	Date acquired	Source
C3Hf/PW	1938	Bittner.
C3Hf/Gs	1958	Gross via Woolley.
CBA/H	1949	Harwell.
Ak_f	1945	Furth via Engelbreth-Holm.

ice-cold saline with a previously chilled mortar and pestle so as to make a 20 per cent homogenate. This was centrifuged in a Servall centrifuge at 1400 *g* for 15 minutes in the cold room. The supernatant fluid was then centrifuged at 7000 *g* for 10 minutes and the final supernatant fluid filtered through a chilled Selas O2 porosity filter candle under vacuum pressure. The extracts were either used immediately or sealed in 2 ml. glass ampoules and stored at −79° C.

The extracts were prepared from thymus, spleen and lymph nodes of mice with either spontaneous or induced leukaemias: the spontaneous leukaemias were obtained from our own colony of Ak_f and from AKR mice received from other laboratories; the induced leukaemias were obtained from C3Hf/Gs mice inoculated by Dr. Gross with his potent Passage A filtrate (Gross, 1957*b*).

Inoculation procedures.—Filtrates were generally injected into newborn mice less than 16 hours after birth, each baby mouse receiving 0·1 ml. by the intraperitoneal route, the needle first traversing the thigh muscles to avoid leakage. When Passage A filtrate was used however, suckling mice up to 14 days of age were injected, the volume of the inoculum being varied according to age: at 1 and 2 days 0·1 ml. was given, at 3 and 4 days 0·2 ml. and at 5 to 14 days 0·3 ml. Adult mice inoculated with filtrates received 0·5 ml. intraperitoneally daily for 3 days. Control mice were usually littermates identified by tail-clipping; they received either saline of filtrates heated to 56° C. for 30 minutes.

Transplantation of leukaemias.—For transplantation of leukaemias, cell suspensions were prepared from fresh leukaemic spleen by teasing out with forceps in a dish of saline. The cells were washed in saline to the desired volume so that 0·5 ml. of the suspension contained 30 to 50 million cells. This amount was injected intraperitoneally into adult mice of the strains tested for transplantability. The cells of each case of leukaemia to be transplanted were injected into 3 to 5 mice of each strain.

Induction of immune tolerance.—For induction of specific immune tolerance to Ak tissues, cell suspensions from thymus or spleen of one-month-old healthy Ak_f donors were prepared by teasing out in buffered Ringer phosphate solution, washing twice, and resuspending in the buffered solution so that 0·05 ml. of the suspension contained 5 to 7 million cells. This amount of the freshly prepared suspension was injected into the anterior facial vein of newborn C3Hf/PW mice less than 20 hours after birth.

Skin grafts.—Skin grafts were performed according to the method of Billingham and Medawar (1951) at 6 to 8 weeks of age. The grafted skin was from 2-months-old healthy Ak_f female mice and served as the external indicator of the tolerant state. Only fully tolerant mice, carrying healthy skin grafts were used experimentally.

Histology.—Tissues for histological examination were fixed in Bouin's fluid and stained in haematoxylin and eosin.

RESULTS

Detailed results are presented in Tables II to VIII. The salient features of these tables may be summarized as follows.

Incidence of spontaneous leukaemia (Table II)

Only the Ak_t strain had a high incidence of spontaneous leukaemia during the fourteen month period of observation. The final figures for the full life span of the low-leukaemic strains may well be rather different.

TABLE II.—*Incidence of Spontaneous Lymphocytic Leukaemia in Untreated Mice of Four Different Strains*

Strain of mice	Number of mice observed for 14 months	Mice with lymphocytic leukaemia		
		Number	Age in months	Per cent
C3Hf/PW	227	2	(12, 14)	Less than 1
C3Hf/Gs	79	0	—	0
CBA/H	121	0	—	0
Ak_t	193	171	Average 8·9	88

Incidence of lymphocytic leukaemia in mice inoculated at birth with cell-free extracts of leukaemic tissues (Table III)

(a) *Controls.*—The saline and heat-inactivated filtrate controls in all cases did not have a significantly different incidence of leukaemia from the completely untreated mice.

TABLE III.—*Incidence of Lymphocytic Leukaemia in Mice Following Inoculation at Birth of Cell-Free Extracts*

Strain	Filtrate given at birth	Number in group	Mice with lymphocytic leukaemia*		
			Number	Age in months	Per cent
C3Hf/PW	Ak leukaemic	68	8	7–14 (average 11)	11·8
	Passage A	45	12	7–10 (average 9)	26·7
	Saline	39	0	—	0
C3Hf/Gs	Passage A	87†	87	2–4 (average 3)	100
	Heated Passage A	15	0	—	0
CBA/H	Ak leukaemic	71	5	6–15 (average 11)	7
	Saline	40	0	—	0
Ak_t	Ak leukaemic	75	51	3–6 (average 4·7)	78·7
			8	7–12	
	Passage A	15†	15	3–6 (average 4·1)	100
	Saline	34	31	7–11 (average 9·6)	91

* Survivors are over 12 to 14 months of age.
† These mice were injected 1 to 14 days after birth.

(b) *Filtrates from Ak leukaemic tissues.*—Filtrates from leukaemic Ak mice had a marked effect only in Ak_t mice where the average age of onset of the disease was reduced from about 9 months to about 5 months. This effect must be regarded as an acceleration of the leukaemogenic process. The total incidence of the disease in the inoculated group was slightly lower than in the completely untreated group as a result of a higher mortality from non-leukaemic causes in inoculated mice.

A slightly increased incidence of leukaemia was observed in C3Hf/PW and CBA/H mice following injection of Ak leukaemic filtrates at birth.

Thirty-one separate Ak filtrates were used in these experiments but only 8 were associated with leukaemogenic activity in C3Hf/PW and CBA/H mice (Table IV). Five filtrates each produced leukaemia in 2 mice and in 3 cases the 2 mice came from the same litter. Sixteen of the filtrates used were responsible for the development of early leukaemias in inoculated Ak_t mice.

(c) *Passage A filtrate.*—Injection of Passage A filtrate at or soon after birth gave 100 per cent incidence of leukaemia in both C3Hf/Gs and Ak_t mice as early as 2 to 4 months. This filtrate increased the incidence of leukaemia following injection into newborn C3Hf/PW mice to about 27 per cent the disease developing between 7 and 10 months of age.

TABLE IV.—*Activity of Filtrates from Tissues of Ak Mice with Spontaneous Leukaemia*

	Recipients (inoculated at birth)									
	Low-leukaemic C3Hf/PW and CBA/H mice					High-leukaemic Ak_t mice				
				Leukaemia incidence					Incidence of early (3-6 months) leukaemia	
Source of filtrates	Number of active filtrates	Number of mice inoculated	Number of susceptible litters	Number	Per cent	Number of active filtrates	Number of mice inoculated	Number of susceptible litters	Number	Per cent
Ak_t (Pollards Wood)	5/15	73	6/26	7	9·6	10/14	40	15/18	27	67·5
AKR (Manchester) .	1/8	32	2/10	2	6·3	3/5	17	3/5	11	64·7
AKR (Gif-sur-Yvette)	1/4	19	1/4	2	10·5	2/3	9	2/3	7	77·8
AKR (Paris) . .	1/4	15	1/4	2	13·3	1/2	9	2/3	6	66·7
Total . .	8/31	139	10/44	13	9·4	16/24	75	22/29	51	68·0

Transplantability of the leukaemias

Seven of the leukaemias produced in C3Hf/PW after injection of filtrates of Ak leukaemic tissues were tested for transplantability in Ak_t and C3Hf/PW mice. All were transplantable to C3Hf/PW and 3 to Ak mice as well. Twenty-four Passage A induced leukaemias in C3Hf/Gs mice were transplanted. All were transplantable to C3Hf/Gs and 15 also to Ak_t. Three leukaemias arising in CBA/H mice were transplantable only to CBA/H mice.

The development of a potent Ak filtrate (Table V)

Filtrates from Ak leukaemic tissues was passed through serial cell-free inoculations into 4 successive generations of newborn Ak mice. In each passage, the first Ak mouse that developed leukaemia was used as donor for the preparation

TABLE V.—*The Incidence of Lymphocytic Leukaemia in C3Hf/Gs Mice Following Inoculation of Ak Leukaemic Filtrates Passaged Serially Through Ak Mice*

Filtrate given at birth	Number of C3Hf/Gs mice inoculated	C3Hf/Gs mice with lymphocytic leukaemia		
		Number	Age in months	Per cent
Ak leukaemic (from spontaneous case)	14	0	—	0
Ak leukaemic (after 3 passages in Ak mice)	11	5	4–10 (average 7·4)	45·5
Ak leukaemic (after 4 passages in Ak mice)	16*	11	3–6 (average 5·1)	68·8

* These mice were inoculated between 1 and 5 days after birth.

of the next passage-extract. The final filtrate considerably increased the total incidence of the disease and accelerated the average age of onset when inoculated into C3Hf/Gs mice at or soon after birth.

TABLE VI.—*Incidence of Lymphocytic Leukaemia in Mice Following Inoculation of Cell-Free Extracts at 4 to 8 Weeks of Age*

Strain	Filtrate given at 4 to 8 weeks	Number in group	Mice with lymphocytic leukaemia*		
			Number	Age in months	Per cent
C3Hf/PW	Ak leukaemic	34	0	—	0
C3Hf/Gs	Ak leukaemic	18	0	—	0
	Passage A	20	12	4–8 (average 6·8)	60
Ak_i	Ak leukaemic	18	15	8–11 (average 8·9)	83·3

* Survivors are over 12 to 14 months of age.

The incidence of lymphocytic leukaemias in mice inoculated as adults with cell-free extracts of leukaemic tissues (Table VI)

Cell-free filtrates of leukaemic tissues of Ak mice were not capable of inducing leukaemias in adult C3Hf/Gs or C3Hf/PW mice. Though a high number of Ak_i mice developed leukaemia following injection at 4 to 8 weeks of age with filtrates of Ak leukaemic tissues, the total incidence and average age of onset were almost the same as in untreated Ak_i mice. It must, therefore, be assumed that the filtrates had no noticeable effect when injected into adult mice.

Passage A filtrate given to C3Hf/Gs at 4 to 8 weeks of age did produce some leukaemias but not as efficiently as the same filtrate given at or soon after birth.

The incidence of lymphocytic leukaemia in C3Hf/PW mice tolerant to Ak_i and inoculated as adults with cell-free extracts of Ak leukaemic tissues (Table VIII)

An intravenous injection of healthy Ak_i spleen or thymus cells into newborn C3Hf/PW mice induced long-lasting tolerance to Ak_i skin grafts in up to 90 per cent of the mice (Table VII). Transplants of Ak leukaemic cells grew progressively in all cases in tolerant C3Hf/PW mice but never in non-tolerant mice. On further transplantation to groups of non-tolerant C3H and of Ak_i mice the trans-

TABLE VII.—*Tolerance to Ak Skin Graft in C3Hf/PW Mice Inoculated at Birth with Ak Spleen or Thymus Cells*

Ak cells given at birth		Number of mice in group	Number of mice fully tolerant
Number	Type		
5–7 million	Spleen	143	128 (89%)*
5–7 million	Thymus	161	137 (85%)*
0	—	57	0 (0%)†

* Ak skin graft intact to date (over 12 months).
† Ak skin graft rejected in 11 ± 1 days.

plants were successfully established only in Ak mice thus showing that the genetic constitution of the transplanted leukaemia was still Ak.

TABLE VIII.—*Incidence of Lymphocytic Leukaemia in C3Hf/PW Mice Tolerant to Ak Following Inoculation of Ak Leukaemic Extracts at 4 to 6 Weeks of Age*

Intravenous injection at birth	Filtrate given at 4 to 6 weeks	Number of mice in group	Mice with leukaemia		
			Number	Age	Per cent
Ak spleen cells	Ak leukaemic	31	0	—	0
Ak thymus cells	„ „	28	0	—	0
Ak spleen or thymus cells	None	20	0	—	0

No leukaemia occurred in C3Hf/PW mice made tolerant to Ak and injected 1 month after birth with cell-free filtrates from Ak leukaemic tissues (Table VIII). Sixteen of the mice in this group received at least one of the 8 filtrates that was associated with leukaemogenic activity following inoculation into newborn C3Hf/PW or CBA/H mice.

DISCUSSION

The work described here confirms the results obtained by Gross (1958*b*) and others (Woolley and Small, 1956 ; Furth *et al.*, 1956 ; Dulaney *et al.*, 1957 ; Hays and Beck, 1958 ; Kassel and Rottino, 1959).

Cell-free extracts of tissues of Ak mice with spontaneous leukaemia are clearly capable of leukaemogenic activity. In the high-leukaemia Ak strain and in the (AKRXC3Hf)F_1 hybrids, there is little or no change in leukaemia incidence but an acceleration of the onset of the disease following inoculation of extracts at birth (Rudali, Duplan and Latarjet, 1957 ; Law, Dunn and Boyle, 1955). The leukaemic agent has been shown to be present in young healthy Ak mice (Gross, 1951, 1953), so that inoculation of leukaemic extracts into these mice must be assumed to increase the quantity of agent already present in the host. Acceleration of leukaemia may thus be explained on a purely quantitative basis. Extracts from normal tissues of low-leukaemic C3H mice failed to be associated with leukaemogenic activity following inoculation into newborn C3H mice (Gross, 1955*b*). These mice may, therefore, not carry the leukaemic agent, and leukaemia occurring as a result of inoculation of Ak leukaemic extracts must presumably have been induced by the agent. It is true, however, that the disease occurs spontaneously in low-leukaemic strain mice (Law, 1957) and two cases have been diagnosed in our colony of C3Hf/PW kept under observation for only 14 months.

These two cases still have to be explained. It is possible that some of the C3H mice carry the agent, but that our methods are not sensitive enough to demonstrate its presence ? If this is so, does the inoculation of leukaemic filtrate at birth simply accelerate the onset of the disease in mice that would develop it if they lived long enough ? Some agent must be present in C3H mice since Gross claims to have activated it by X-irradiation (Gross, 1958*a*). The point at issue is whether this agent is or is not identical to that present in high-leukaemia strains.

Filtrates from Ak leukaemic tissues have been shown to vary considerably in their activity. Many of them were associated with the development of early leukaemia in Ak mice, but only few produced leukaemia in C3Hf/PW mice (Table IV). Gross developed his potent Passage A filtrate (Gross, 1957*b*) by selecting an active Ak leukaemic extract and passing it through serial cell-free inoculation of newborn C3Hf/Gs mice. In the work reported here, a potent extract was obtained in a similar way but in a relatively shorter period of time, the extracts being passed through successive generations of newborn Ak mice instead of C3Hf/Gs mice (Table V). No doubt this procedure must select and concentrate active agent, since dilution of the final potent extract reduces the total incidence and delays the average age of onset of leukaemia in inoculated mice (Gross and Dreyfuss, 1959).

Subline differences exist in respect to susceptibility to leukaemogenesis by any given extract as was pointed out by Gross (1955*a*) and Woolley and Small (1957). In this study, a valid comparison can be made of the response of 2 sublines of C3H mice, both originally obtained from Bittner, since both were injected simultaneously with the same filtrate, Passage A. It is obvious from Table III that the C3Hf/Gs subline is by far the more sensitive.

Only newborn mice were susceptible to the leukaemogenic activity of filtrates prepared from tissues of Ak mice with spontaneous leukaemia. This may be due to :

(1) A state of immunological unresponsiveness ;
(2) a concentration effect ;
(3) factors, other than immunological, inherent to the particular stage of development of the animals at birth.

(1) It has been suggested that immunological tolerance is the reason why only newborn mice are susceptible to an agent recently isolated from a foreign strain (Harris, 1958 ; Barnes *et al.*, 1959). In this study, a state of acquired tolerance in C3H mice to transplantation antigens of Ak mice did not appear to give tolerance to the Ak leukaemic agent (Table VIII). The mice were fully susceptible to grafts of Ak leukaemic cells but not to the leukaemogenic activity of the Ak agent. For the induction of tolerance, normal Ak cells, which according to Gross (1953, 1959) contain the agent, were injected intravenously at birth, and yet no leukaemia developed. Perhaps the quantity of agent in 5 million normal Ak cells is not sufficient for leukaemogenesis, or perhaps the agent is intracellular, antigenically distinct and must be introduced in the free state in newborn animals.

(2) Simple quantitative factors may serve to explain the age susceptibility to the leukaemogenic effect of cell-free filtrates. For any active filtrate, the concentration of agent present in the newborn after inoculation must be relatively far greater than that which can be attained in an adult mouse. Augmentation of " infectivity " presumably occurs as a result of selection of the most active extract

after serial passage through successive generations of mice of any one strain. A far greater concentration of active agent can then be achieved following inoculation into suckling or young adult mice. This may explain why acceleration of leukaemia in Ak mice by Ak filtrate can only occur when the filtrate is given at birth, and why, on the other hand, Passage A can accelerate Ak leukaemia when given to older Ak mice or can produce leukaemia after inoculation into young adult C3Hf/Gs mice.

(3) Shubik has pointed out that the response of newborn mice to filtrates should also be viewed as a response of a sensitive biological system to carcinogenesis (Pietra, Spencer and Shubik, 1959). He reports a high incidence of early malignant lymphomas in a relatively insusceptible strain of mice following the inoculation at birth of a single low dose of chemical carcinogen.

All the leukaemias developing in our inoculated mice were lymphoid with thymus involvement resembling typical spontaneous lymphomas in Ak mice. They were all transplantable to mice of the recipient line, and many were transplantable to both donor and recipient strains. This confirms similar observations made by Furth *et al.* (1956) and Gross (1958*c*). No other tumours, nor tumours characteristic of polyoma virus infection (Stewart *et al.*, 1957) were seen in any of the mice of the present series in contrast to the results of other workers in this field (Stewart, 1955 ; Law *et al.*, 1955 ; Salaman, 1959). Salaman, for instance, recorded the occurrence of 40 tumours (but no leukaemia) among 15 out of 23 C3Hf/Bi mice inoculated at birth with leukaemic filtrate. However, the filtrates were prepared from leukaemias that had already been transplanted three times by cell graft through AKR mice. Gross has stressed that as far as leukaemogenesis by cell-free extracts is concerned, the source of material should either be a spontaneous leukaemia or one induced with leukaemic extracts rather than a transplanted leukaemia (Gross, 1956, 1958*b*). Some supporting evidence for this claim was obtained by Kassel and Rottino (1959) who showed that extracts prepared from transplanted AKR leukaemic tissues resulted in the development of parotid tumours, adrenal tumours, osteosarcomas and fibromyxomas but no leukaemia.

It would be pure speculation to attempt, on the basis of the present results, to identify the agent in leukaemic filtrates as a virus or as a genic complex derived from either neoplastic or potentially neoplastic cells. An increase in leukaemia incidence, *per se*, does not establish the existence of a virus-mediated mechanism. Final identification of the agent must await further investigations of its biological and physicochemical properties.

SUMMARY

1. Newborn or adult mice of the low-leukaemic strains C3Hf/PW, C3Hf/Gs, CBA/H, and of the high-leukaemic strain Ak_i were inoculated intraperitoneally with cell-free extracts prepared from tissues of Ak mice with spontaneous leukaemia, and of C3Hf/Gs mice with leukaemias induced by leukaemic filtrate, Passage A.

2. Ak leukaemic filtrates inoculated into newborn low-leukaemic strain mice resulted in leukaemia in 11·8 per cent of 68 C3Hf/PW mice at 7 to 14 months and 7 per cent of 71 CBA/H mice at 6 to 15 months. No leukaemia occurred when these filtrates were inoculated into 34 adult C3Hf/PW mice or 18 adult C3Hf/Gs mice.

3. Passage A filtrate resulted in 26·7 per cent leukaemias at 7 to 10 months when inoculated into 45 newborn C3Hf/PW mice and 100 per cent leukaemia at 2 to 4 months following inoculation into 87 one- to 14-days-old C3Hf/Gs mice. The same filtrate produced 60 per cent leukaemias when inoculated into 20 one- to 2-months-old C3Hf/Gs mice.

4. Acceleration of the onset of leukaemia to 3 to 6 months occurred in 68 per cent of 75 Ak mice inoculated at birth with Ak leukaemic filtrates but in none of 18 Ak mice inoculated at 1 to 2 months of age. Passage A filtrate accelerated leukaemia in 100 per cent of 15 Ak mice inoculated between 1 and 14 days of age.

5. Transplantation of some of the leukaemias produced in C3H mice revealed some ambivalence: all were transplantable to C3H mice but many were also transplantable to mice of the original donor Ak strain.

6. Increase in potency of an Ak leukaemic filtrate was evident after 4 serial cell-free inoculations of newborn Ak mice. The final filtrate was associated with the development of leukaemia at 3 to 6 months in 68·8 per cent of 16 C3Hf/Gs mice inoculated between 1 and 5 days.

7. No leukaemia developed in 79 C3Hf/PW mice which received at birth an intravenous injection of normal spleen and thymus cells from healthy young adult Ak mice. Fifty-nine of these mice received, in addition, intraperitoneal injections of Ak leukaemic filtrate at 4 to 8 weeks of age. All the mice in this group were fully tolerant of Ak skin grafts.

8. Only lymphocytic leukaemias or lymphoid tumours confined to the thymus were seen in mice of the present series. No other tumours such as those described by other workers in this field have appeared.

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REFERENCES

Barnes, D. W. H., Ford, C. E., Ilbery, P. L. T., Jones, K. W. and Loutit, J. F.—(1959) *Acta Un. int. Cancr.* **15**, 544.

Billingham, R. E. and Medawar, P. B.—(1951) *J. exp. Biol.*, **28**, 385.

Dulaney, A. D., Maxey, M., Schilling, M. G. and Goss, M. F.—(1957) *Cancer Res.*, **17**, 809.

Friend, C.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, 522.

Furth, J., Buffett, R. F., Banasiewicz-Rodriguez, M. and Upton, A. C.—(1956) *Proc. Soc. exp. Biol., N.Y.*, **93**, 165.

GRAFFI, A.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, 540.
GROSS, L.—(1951) *Proc. Soc. exp. Biol., N.Y.*, **76**, 27.—(1953) *Acta haemat.*, **10**, 18.—(1955*a*) *Proc. Soc. exp. Biol., N.Y.*, **88**, 64.—(1955*b*) *Ibid.*, **88**, 362.—(1956) *Acta haemat.* **15**, 273.—(1957*a*) *Ann. N.Y. Acad. Sci.*, **68**, 501.—(1957*b*) *Proc. Soc. exp. Biol., N.Y.*, **94**, 767.—(1958*a*) *Acta haemat.*, **19**, 353.—(1958*b*) *Cancer Res.*, **18**, 371.—(1958*c*) *Proc. Soc. exp. Biol., N.Y.*, **97**, 300.—(1959) *Ibid.*, **100**, 325.
Idem AND DREYFUSS, Y.—(1959) *Proc. Amer. Ass. Cancer Res.*, **3**, 24.
HARRIS, R. J. C.—(1958) *J. chron. Dis.*, **8**, 58.
HAYS, E. F. AND BECK, W. S.—(1958) *Cancer Res.*, **18**, 676.
KASSEL, R. AND ROTTINO, A.—(1959) *Ibid.*, **19**, 155.
LAW, L. W.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, 616.
Idem, DUNN, T. B. AND BOYLE, P. J.—(1955) *J. nat. Cancer Inst.*, **16**, 495.
MILLER, J. F. A. P.—(1959) Proceedings of the seventh European Congress of Haematology, London, *Acta haemat.* in press.—(1960) *Brit. J. Cancer*, **14**, 93.
PIETRA, G., SPENCER, K. AND SHUBIK, P.—(1959) *Nature*, **183**, 1689.
RUDALI, G., DUPLAN, J. F. AND LATARJET, R.—(1957) *Bull. Ass. franç. Cancer*, **44**, 440.
SALAMAN, M. H.—(1959) *Brit. J. Cancer*, **13**, 76.
SCHWARTZ, S. O. AND SCHOOLMAN, M. H.—(1959) *Blood*, **14**, 279.
STEWART, S. E.—(1955) *J. nat. Cancer Inst.*, **15**, 1391.
Idem, EDDY, B. E., GOCHENOUR, A. M., BORGESE, N. AND GRUBBS, G. E.—(1957) *Virology*, **3**, 380.
WOOLLEY, G. W. AND SMALL, M. C.—(1956) *Cancer*, **9**, 1102.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, 533.

STUDIES ON MOUSE LEUKAEMIA. THE ROLE OF THE THYMUS IN LEUKAEMOGENESIS BY CELL-FREE LEUKAEMIC FILTRATES

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TOTAL thymectomy markedly reduces the high incidence of spontaneous lymphomas in certain strains of mice (McEndy, Boon and Furth, 1944 ; Law and Miller, 1950*a*). A similar effect is seen in mice in which the disease can be induced by ionizing radiations (Kaplan, 1950), carcinogenic hydrocarbons (Law and Miller, 1950*b*) or the inoculation at birth of cell-free leukaemic extracts (Gross, 1959 ; Levinthal, Buffett and Furth, 1959 ; Miller, 1959*a*, 1959*c*). Thymectomy has no effect on radiation-induced myeloid leukaemia in RF mice (Upton *et al.*, 1958) suggesting that the leukaemogenic influence of the thymus is specific for lymphoid tissues. There are no differences between thymectomized and control groups of mice in weight curves, breeding behaviour or susceptibility to common laboratory infections. Reduction of leukaemia incidence by thymectomy does not seem, therefore, to be related to other factors affecting the general health of the animals (Law and Miller, 1950*a*, 1950*b*).

Subcutaneous grafts of autologous or isologous thymus in thymectomized mice restore the potentiality of developing the disease, whether this be spontaneous, induced by carcinogen (Law and Miller, 1950*a*, 1950*b*) or by irradiation (Kaplan *et al.*, 1956).

The work to be reported here shows that while thymectomy prevents the development of the disease following inoculation of leukaemic filtrate, thymus grafting as late as 6 months after thymectomy restores the potentiality for leukaemia development in mice inoculated at birth with cell-free filtrates. A preliminary account of this work has been given elsewhere (Miller, 1959*b*).

MATERIALS AND METHODS

The strains C3Hf/PW, C3Hf/Gs, CBA/H and Ak_i (Miller, 1960) were used. Filtrates were prepared in the same way and from the same sources as described elsewhere (Miller, 1960). The route of inocualtion and the doses given were also the same.

Thymectomy was usually performed at 4 weeks of age. The thymus was removed by suction through an incision in the neck and thoracic wall extending to the level of the second rib. Thymus grafting was performed by introducing one whole thymus with a trocar and cannula into the subcutaneous tissues of the right or left axilla. Donor and recipient mice were always of the same sex and the age of the donor varied from 1 to 30 days according to the experiment. Strict asepsis was observed during the grafting procedure.

EXPERIMENTAL

Three experiments were set up as follows :

Experiment I.—Mice of all 4 strains were inoculated at birth with a filtrate of leukaemic tissue. At 4 weeks of age about half the inoculated mice were thymectomized. Some of the thymectomized mice received a further inoculation of leukaemic filtrate after thymectomy.

Experiment II.—Normal mice of the C3Hf/Gs strain were thymectomized at about 4 weeks of age and received from 1 day to 1 month after thymectomy a subcutaneous thymus graft. The thymuses were obtained either from normal newborn C3Hf/Gs mice (Group I) or from C3Hf/Gs mice between 5 and 30 days of age that had themselves been inoculated at birth with Passage A filtrate (Group II).

Experiment III.—Two groups of mice were studied : the first was inoculated with leukaemic filtrates soon after birth and thymectomized 4 weeks later, and the second was first thymectomized at about 4 weeks and inoculated with leukaemic filtrate just after thymectomy. The mice in both groups were grafted subcutaneously with day-old thymuses from normal C3Hf/Gs mice. These grafts were performed from 1 day to 6 months after thymectomy in the first group, and from 1 to 2 months after thymectomy in the second group.

RESULTS

Experiment I.—The development of lymphocytic leukaemia in thymectomized mice inoculated at birth with leukaemic filtrates (Table I)

Thymectomy prevented leukaemia in all but 3 Ak_1 mice of the strains tested. This was particularly striking in the C3Hf/Gs strain where Passage A inoculation into mice at or soon after birth was followed by 100 per cent leukaemias in non-thymectomized littermates. Further inoculation of extracts after thymectomy did not raise the incidence of leukaemia in thymectomized hosts. The incidence

TABLE I.—*Experiment I.—Incidence of Lymphocytic Leukaemia in Thymectomized Mice*

Strain	Filtrate given at birth	Thymus	Number in group	Mice with lymphocytic leukaemia*		
				Number	Age in months	Per cent
C3Hf/PW	Ak leukaemic or Passage A	Intact	113	20	7–14	17·7
		Removed†	59	0	—	0
C3Hf/Gs	Passage A	Intact	45	45	2–4	100
		Removed†	38	0	—	0
CBA/H	Ak leukaemic	Intact	71	5	6–15	7
		Removed	28	0	—	0
Ak_1	Ak leukaemic	Intact	69	47	3–6	78·2
				7	9–10	
		Removed	62	3	3, 6 and 10	4·8

* Survivors are over 12 months of age.

† Some of these mice received further inoculations of leukaemic filtrate after thymectomy.

of spontaneous leukaemia in untreated mice of the strains used here has been reported elsewhere (Miller, 1960).

Experiment II.—The development of lymphoid tumours in thymuses grafted to thymectomized mice (Table II)

The fate of thymuses grafted to normal hosts was examined. Thymuses taken from normal donors did not develop lymphoid tumours (Group I). On the other hand, thymuses taken from donors which had themselves been inoculated with leukaemic extracts at birth became malignant in some of the uninoculated hosts three to five months after grafting (Group II).

TABLE II.—*Experiment II.—Incidence of Lymphoid Tumours in Thymuses Grafted to Thymectomized C3Hf/Gs Mice*

	Donor C3Hf/Gs*		Host C3Hf/Gs†		Mice with lymphoid tumours		
Group	Treatment given at birth	Age (in days) of thymus when grafted	Number of mice grafted	Age of host at grafting (months)	Number	Age in months	Per cent
I	None	1	20	1–2	0	—	0
II	Passage A	5–9	10	2	3	5–7	30
		10–30	17	2	10	5–6	58·4

* Donors in Group I were not inoculated. Donors in Group II received Passage A filtrate at birth.

† Hosts in either group were not inoculated with filtrates. They were all thymectomized at 1 month of age.

Experiment III.—The development of lymphoid tumours in normal thymuses grafted to thymectomized hosts inoculated with leukaemic filtrate (Table III)

The fate of thymuses from normal donors was studied in inoculated thymectomized hosts. About half these thymuses developed lymphoid tumours when introduced as late as 6 months after thymectomy and almost all became malignant when grafted within three months after thymectomy (Group I). Lymphoid tumours were diagnosed from 2 to 4 months after grafting.

Thymuses introduced into hosts inoculated after thymectomy also developed lymphoid tumours, but only in one-third of the mice (Group II).

TABLE III.—*Experiment III.—Incidence of Lymphoid Tumours in Thymuses Grafted to C3Hf/Gs Mice Inoculated with Leukaemic Filtrates*

			Age of hosts when grafted (months)†	Number of mice	Mice with lymphoid tumours		
Group*	Strain	Filtrate given			Number	Age in months	Per cent
I	C3Hf/Gs	Passage A	1	10	10	3–4	100
			2–3	18	15	3–5	83·3
			3–4	12	9	5–8	75
			7	11	6	9–11	54·5
II	C3Hf/Gs	Passage A	2–3	9	3	7–8	33·3

* Mice in Group I were inoculated at birth and later thymectomized. Mice in Group II were thymectomized at 24 days and inoculated between 35 and 40 days.

† All the mice were thymectomized at 24 to 30 days of age and received day-old thymuses from uninoculated healthy isologous mice.

Transplantation

The thymomas could be transplanted in all cases to untreated 1- to 2-months-old C3Hf/Gs mice.

In the majority of cases, the tumours in Experiments II and III appeared at first to be confined to the subcutaneous spaces where the thymus had been grafted. At this stage the spleens from these animals did not produce leukaemia after transplantation. Later, dissemination took place and generalized leukaemia became evident in the thymus grafted hosts. These transplantation studies are still in progress.

DISCUSSION

Our results confirm the previous findings that thymectomy prevents the development of lymphomas in mice inoculated with leukaemic filtrates (Gross, 1959 ; Levinthal *et al.*, 1959 ; Miller, 1959*a*). They show, in addition, that the potentiality for leukaemia development is still present in the inoculated thymectomized host, and that normal thymuses can express this potentiality when grafted to a subcutaneous site in the inoculated thymectomized host as late as 6 months after thymectomy.

Thymectomy might prevent the development of leukaemia following inoculation of leukaemic filtrates by effecting removal of :

(1) The source of the leukaemic agent ;
(2) the site of multiplication of the agent ;
(3) the cells most susceptible to leukaemic transformation ;
(4) the source of a humoral factor involved in leukaemogenesis.

(1) In the present experiments, normal thymuses grafted in thymectomized inoculated hosts as late as 6 months after thymectomy developed lymphoid tumours. It cannot be maintained, therefore, that thymectomy prevents leukaemia development in inoculated mice by removing either the source of the leukaemic agent or the site where the agent is principally stored. This does not exclude the possibility that the thymus may contain some of the agent in inoculated mice (Gross, 1959) but it does exclude the suggestion that the thymus is the only source of the agent. It follows that the agent might be recoverable from tissues of inoculated hosts up to 6 or more months after thymectomy. Experiments are now in progress to determine whether this can be done.

(2) It is possible that the leukaemic agent must reach a critical concentration to produce leukaemia in its host. If so, the fact that no lymphoid tumour occurred in inoculated thymectomized mice until a thymus graft was introduced can be interpreted to mean that the agent multiplies satisfactorily only in thymus tissue.

(3) In every case of leukaemia following the inoculation of leukaemic filtrates the thymus was involved, and in some cases it was the sole organ involved. The results obtained in Experiment II show that cells capable of leukaemic transformation are present in the thymus as early as 5 to 10 days after the inoculation of leukaemic filtrates. Removal of such a potentially malignant focus would thus prevent the development of the disease. Thymus involvement in C58 mice and in DBA/2 mice painted with methylcholanthrene is, however, very rare (Law and Miller, 1950*a*, 1950*b*) and yet the disease can be prevented by thymectomy. It

is difficult to assume that such a procedure, in this case, simply acts by removing the cells most susceptible to leukaemic transformation.

The evidence obtained from the present work is not sufficient to decide for or against any or both of these last two possible explanations. The fact that thymus grafted to inoculated thymectomized hosts develops lymphoid tumours can be taken as supporting evidence for either of these hypotheses.

(4) Our results do not exclude the possibility that a humoral factor might be involved in leukaemogenesis in hosts that are conditioned either by irradiation, or chemical carcinogens, or the inoculation of leukaemic filtrates. The possibility of a non-cellular factor from the thymus exerting an influence in the leukaemogenic process has been stressed by Law (Law and Miller, 1950*a* ; Law and Potter, 1956), and Metcalf (1958), who suggests that this factor is his thymic lymphocytosis stimulating factor which he has shown to stimulate lymphocyte proliferation in the mouse.

SUMMARY

1. The effect of thymectomy and thymus grafting on the leukaemogenic activity of cell-free leukaemic extracts has been investigated.

2. No leukaemia occurred in 59 C3Hf/PW, 38 C3Hf/Gs and 28 CBA/H mice inoculated at birth with leukaemic filtrates and thymectomized at 4 weeks of age. The incidence of leukaemia in non-thymectomized inoculated mice of the same strains was 17·7 per cent of 113 C3Hf/PW mice, 100 per cent of 45 C3Hf/Gs mice and 7 per cent of 71 CBA/H mice.

3. Only 3 leukaemias occurred in a group of 62 thymectomized Ak_1 mice which were inoculated at birth with leukaemic filtrates. The incidence of the disease in 69 non-thymectomized inoculated Ak_1 mice was 78·2 per cent the majority of the mice succumbing between 3 and 6 months of age.

4. Thymuses from normal day-old C3Hf/Gs mice grafted to normal adult thymectomized C3Hf/Gs mice did not develop lymphoid tumours or induce leukaemia in their hosts. From 30 to 60 per cent of thymuses from 5 to 30 days old C3Hf/Gs mice inoculated at birth with leukaemic filtrate (Passage A) developed lymphoid tumours when grafted to normal uninoculated thymectomized C3Hf/Gs mice.

5. Thymuses from normal, uninoculated, day-old, C3Hf/Gs mice were grafted to adult thymectomized C3Hf/Gs mice which had themselves been inoculated at birth with Passage A filtrate. From 50 to 100 per cent of these thymuses developed lymphoid tumours in inoculated mice grafted from 1 day to as late as 6 months after thymectomy.

6. The implications of these results are discussed. It is concluded that the potentiality for leukaemia development is still present in inoculated thymectomized hosts for many months after thymectomy and that thymus grafting at any time will express this potentiality in full.

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REFERENCES

GROSS, L.—(1959) *Proc. Soc. exp. Biol., N.Y.*, **100**, 325.
KAPLAN, H. S.—(1950) *J. nat. Cancer Inst.*, **11**, 83.
Idem, CARNES, W. H., BROWN, M. B. AND HIRSCH, B. B.—(1956) *Cancer Res.*, **16**, 422.
LAW, L. W. AND MILLER, J. H.—(1950*a*) *J. nat. Cancer Inst.*, **11**, 253.—(1950*b*) *Ibid.*, **11**, 425.
Idem AND POTTER, M.—(1956) *Proc. nat. Acad. Sci.*, **42**, 160.
LEVINTHAL, J. D., BUFFETT, R. F. AND FURTH, J.—(1959) *Proc. Soc. exp. Biol., N.Y.*, **100**, 610.
MCENDY, D. P., BOON, M. C. AND FURTH, J.—(1944) *Cancer Res.*, **4**, 377.
METCALF, D.—(1958) *Ann. N.Y. Acad. Sci.*, **73**, 113.
MILLER, J. F. A. P.—(1959*a*) *Nature*, **183**, 1069.—(1959*b*) *Ibid.*, **184**, 1809.—(1959*c*) Proceedings of the seventh European Congress of Haematology, London, *Acta haemat.*, in press.—(1960) *Brit. J. Cancer*, **14**, 83.
UPTON, A. C., WOLFF, F. F., FURTH, J. AND KIMBALL, A. W.—(1958) *Cancer Res.*, **18**, 842.

STUDIES ON FLUID MEDIA FOR THE CULTIVATION OF MOUSE ASCITES TUMOUR CELLS *IN VITRO*

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ASCITES tumours in experimental animals are essentially almost pure populations of suspended tumour cells multiplying in peritoneal exudate. Amoebocytes and shed peritoneal cells are usually present in relatively low numbers. The suitability of these tumours for quantitative studies on malignant cells has long been realised. As a measure of chemotherapeutic activity, Lettré (1941, 1950) used differences in the survival times of control and treated mice injected with uniform volumes of ascites tumour fluid and reported that increases in body weight of inoculated mice were proportional to increases in numbers of the tumour cells. This latter relation was shown by Klein (1951) to be valid only for inoculations with certain numbers of tumour cells.

The value of Ehrlich ascites tumour cells for quantitative studies on growth and biochemistry has been discussed in detail by Klein (1950, 1951). These particular advantages include ease of serial sampling, low incidence of necrotic cells, uniform nutritive conditions and distribution of cells in the exudate, and direct action of agents on the tumour cells. *In vitro* cultivation of ascites tumour cells in fluid media offers additional advantages. It affords more precise control of the extracellular medium and environment, and direct microscopic observation of the tumour cells *in situ*. A fluid medium supporting multiplication of Ehrlich ascites tumour cells *in vitro* has therefore been devised.

MATERIALS AND METHODS

Mice and tumours.—Ehrlich ascites tumours were maintained in 12- to 15-week-old RIII strain mice. They were propagated by serial intraperitoneal injections of 0·1–0·2 ml. volumes of ascites tumour fluid taken from mice inoculated 6 to 7 days previously. The 10 to 12 mice in each experimental group were inoculated with ascitic fluid from one donor. The mice were given normal laboratory diet and water *ad libitum*.

Tissue culture.—Ascites tumour fluid was withdrawn aseptically from mice inoculated 6 to 7 days previously with tumour cells and killed by dislocation of the neck. Pooled ascitic fluid from the mice of a single inoculation group was used for each individual experiment. A portion of this pooled ascitic fluid was reserved untreated for addition as cell inoculum to the culture medium. The remainder was centrifuged for 10 minutes at about 3000 r.p.m. in a bench centrifuge, the supernatant "ascitic plasma" removed and stored at 4° for later use. In the present context "ascitic fluid" refers to the cell-containing peritoneal exudate

as removed from mice, and "ascitic plasma" to cell-free supernatant obtained by centrifugation.

Earle's buffered saline solution (Earle, 1943) was sterilised by Seitz filtration and adjusted to pH 7·4–7·6. It was used as the solvent for trypsin and hyaluronidase. Trypsin (B.D.H., commercial grade) was made up at a concentration of 1 mg. per ml. and sterilised by Seitz filtration. Hyaluronidase ("Rondase", Evans) was dissolved in sterile saline to give a concentration of 0·5 mg. per ml. Both enzyme solutions were used at pH 7·4–7·6 and prepared freshly for use. Heparin ("Liquemin", Roche) was prepared in 0·85 per cent sodium chloride solution at a concentration of 25 i.u. per ml. It was ampouled in convenient volumes, autoclaved and stored at 4°.

Embryo extract was prepared from mouse embryos of the RIII strain taken at about the 15th day of development. The freed embryos were cut into large fragments, blood removed by repeated washings with Earle's solution and the tissue then finely minced with curved scissors to give an almost homogeneous pulp. This was churned with Earle's solution by means of a coarse pipette and stood at laboratory temperature for 2 to 3 hours before being centrifuged for 5 minutes at 3000 r.p.m. The ratio of tissue to supernatant after centrifugation was approximately 1 : 2.

In vivo ascites tumour fluid is churned to homogeneity by peristaltic and body movements. In this way fresh nutrients are supplied to the tumour cells and their waste products do not accumulate locally. The churning was simulated by cultivating the ascites cells in standard hexagonal roller-tubes which were revolved in a drum at 11 r.p.m. Each tube was loaded with 2·5 ml. of medium. The medium variants were replicated in each experiment. With the exception of the heparin and Earle's solution, all components of the medium, including ascitic fluid, were prepared as soon as possible before use.

After incubation for 24 hours in suitable media the tumour cells tended to be attached loosely to the surface of the roller-tubes, especially adjacent to the angles between the lateral faces. However, they were readily detached by rotation of the tubes between the palms and a suspension of mostly discrete tumour cells was obtained. Macrophages tended to adhere firmly to the glass but a method of virtually complete recovery of all cells was found. The roller-tubes were coated successively with a silicone ("Repelcote", Hopkins and Williams) and 2 per cent paraffin oil in ethyl ether. The tubes were drained and the ether allowed to evaporate. They were then dry-sterilised. Chilling the culture tubes in ice-water, followed by shaking, detached macrophages. Samples for examination were taken from the homogeneous cell suspensions. Dilute saline solutions of crude trypsin were not completely effective for recovery of macrophages from uncoated roller-tubes although the tumour cells themselves were completely dispersed.

EXPERIMENTAL RESULTS

The experiments refer to Ehrlich ascites rumour cells unless otherwise stated.

Aceto-orcein preparations were used to assess the incidence of mitosis and condition of the cultivated cells in the preliminary experiments. Since ascitic plasma itself would be expected to contain all the factors necessary for the growth of the tumour cells it was tested as such. After incubation of whole unmodified ascitic fluid for 24 hours no dividing cells were observed and almost all the tumour

cells were dead and embedded in fibrin clots. The macrophages present survived far better than the carcinoma cells. The tumour cell population of ascitic fluid *in vivo* during the logarithmic phase of growth may be assumed to be near the maximum allowed by the growth-supporting properties of the peritoneal exudate. Warburg and Hiepler (1952) have demonstrated the nutritive poverty of ascites tumour fluid. Because of this and the absence in *in vitro* conditions of renewal of nutrients and removal of waste products by the host, population densities *in vitro* equivalent to those *in vivo* would be expected to exhaust rapidly the medium.

Accordingly a series of experiments was made in which the proportion of tumour cells per unit volume of the media, which consisted of ascitic fluid and plasma, whole or diluted with Earle's solution, was varied. In view of the possible importance of fibrinogen to the tumour cells ascitic plasma containing 1/10th by volume of heparin solution was used as a diluent instead of ascites tumour serum. These media did not support proliferation although in some the great majority of the carcinoma cells remained viable after incubation for 24 hours. Similar media supplemented with embryo extract to provide growth-stimulating factors gave improved but still unsatisfactory results. Further experiments led to the inclusion of trypsin and hyaluronidase in the culture media.

In the definitive experiments the media consisted of heparin, trypsin and hyaluronidase solutions, embryo extract, ascitic plasma and ascitic fluid, combined in this order to avoid formation of fibrin and possible injury to the tumour cells. At each stage the pooled components were thoroughly mixed before the addition of the next component.

Media with varying proportions of ascitic plasma to standard amounts of the other components were tested. It was established that the optimum proportion of ascites plasma, including the ascitic fluid as plasma, was about 40 per cent of the total medium to 60 per cent for the combined saline components. Native horse serum was substituted for ascites plasma in some experiments and found to maintain the tumour cells in good condition but not to promote cell division.

An empirical medium of ascitic plasma 2 parts by volume, embryo extract 2 parts, heparin, trypsin and hyaluronidase each 1 part, and ascitic fluid 1 part, was adopted as a basis for further study. The plasma moiety, including ascitic fluid, was $37\frac{1}{2}$ per cent and the total saline components $62\frac{1}{2}$ per cent by volume. The packed cell volume of ascitic fluid was about 33 per cent in most instances.

To determine the relative importance of the individual components the basic medium was modified by the substitution of Earle's solution for each saline component, including heparin, separately. The effect of increased numbers of tumour cells inoculated into the medium was also determined. The media are listed in Table I and the results of a typical experiment in Table II. The cell population of the ascitic fluid used as inoculum and of the various media after cultivation for 24 hours were determined by Neubauer haemocytometers, using white cell pipettes. Two or more separate preparations were made for each medium. The media were diluted for counting with 0·05 per cent eosin in Earle's solution. Dilute eosin solutions have been used to estimate cell viability by Schrek (1936), Klein (1951), and Klein and Révész (1953). Staining of cells can be taken as an indication of damage but not all stained cells are non-viable (British Empire Cancer Campaign, 1951). Unstained cells are, however, viable.

The results of a typical experiment are given in Table II. In this particular trial the population of the parent ascitic fluid was found to be 105, 750 cells per

TABLE I.—*Composition, Given in Aliquots by Volume, of Experimental Media Differing in Absence of Single Components from the Basic Medium (No. 1)*

Abbreviations used: MX—embryo extract; HP—heparin; HY—hyaluronidase; TR—trypsin; AF—ascitic fluid.

Components of media	Media numbers					
	1	2	3	4	5	6
Ascitic plasma	2	2	2	2	2	1
Embryo extract	2	—	2	2	2	2
Heparin solution	1	1	—	1	1	1
Hyaluronidase solution	1	1	1	—	1	1
Trypsin solution	1	1	1	1	—	1
Ascitic fluid	1	1	1	1	1	2
Earle's solution	—	2	1	1	1	—
Component tested	—	MX	HP	HY	TR	AF

mm.^{3} and that of each medium except No. 6, was calculated to be 13,219 mm.^{3} The incidence of eosin-stained cells was very nearly 1 per cent. Differential counts of tumour and non-tumour cells were not made because of the low incidence of the latter and the inherent margin of error in haematocyte determinations.

The amount of fibrinogen and consequent extent of clotting varied with individual samples of ascitic fluid from different mice. In this instance the medium without heparin clotted only slightly and supported increased growth. Heparin appeared to be inhibitory at the concentration used in the basic medium but it is possible that the replacement of sodium chloride by balanced saline solution had some effect. Heparin has been shown by Heilbrunn and Wilson (1949) to inhibit cell division and to affect the viscosity of protoplasm. Embryo extract, hyaluronidase and trypsin were necessary for appreciable cell multiplication but the incidence of eosin-stained cells in the respective cultures was significantly increased, compared with the basic medium, only in the absence of embryo extract. Doubling the initial population of tumour cells had a definite effect on proliferation. This was possibly due to increased amounts of soluble protective factors liberated

TABLE II.—*Effects on Cell Population of Absence of Individual Components of the Basic Medium after Incubation for 24 hours*

Medium No.	Component tested	Initial population (mm.^{3})	Population after 24 hours (mm.^{3})	Percentage difference after 24 hours	Percentage of eosin-stained cells after 24 hours
1	(Basic Medium)	13,219	16,250	+23	3·3
2	Embryo extract	13,219	11,700	−11·4	11·9
3	Heparin	13,219	18,800	+42·2	7·4
4	Hyaluronidase	13,219	13,925	+5·3	3·8
5	Trypsin	13,219	12,500	−5·4	4·8
6	(Ascitic fluid)	26,438	43,300	+63·7	29·0

by the cells into the medium. The significance of these factors has been discussed previously (Powell, 1957). Apart from the medium lacking embryo extract, increased frequencies of damaged cells were associated with high proliferation rates and resulting exhaustion of media (No. 3 and 6).

The effects of density of population were further investigated by varying the proportions of ascitic fluid to ascitic plasma but maintaining the combined total of these at 37½ per cent of the complete basic medium. The results of a typical

experiment are given in Table III and illustrate the characteristic relation between density of population and cell multiplication and degeneration, respectively. The incidence of eosin-stained cells was also related to the increase in population during the incubation period.

TABLE III.—*Effects of Increasing Initial Cell Populations upon Proliferation in Basic Medium*

The two higher initial populations were calculated on the basis of that determined for the lowest.

Initial population	Population after 24 hours (mm.³)	Percentage difference in population after 24 hours	Percentage of eosin-stained cells after 24 hours
17,614	23,850	+35·9	2·9
35,228	35,000	±	1·8
52,842	47,350	−10·3	24·4

Initial population densities significantly less than 1/8th of the *in vivo* density were found to be unfavourable for rapid multiplication. This was attributed to the inability of the cells in these numbers to produce an adequate concentration of diffusible protective factors in the medium. In general it appeared that in 2·5 ml. of the basic medium a tumour cell population of about 1/8th of the normal range in native ascitic fluid gave a convenient compromise between cell proliferation and degeneration during an incubation period of 24 hours.

Attempts were made to cultivate Sarcoma 37 ascites tumour cells in similar basic medium with homologous ascitic plasma. These attempts failed. However, multiplication rates comparable to those found *in vivo* were obtained with a modified medium in which ascitic fluid constituted 1/8th of the total volume. In this one of the two parts of the embryo extract was replaced with one of tumour cell extract; in all other respects the basic medium was unchanged. To prepare this extract the sedimented cells of centrifuged Sarcoma 37 ascitic fluid were suspended in an amount of Earle's solution equal in volume to that of the supernatant plasma removed and the suspension incubated for 3 hours at 37°. The cell suspension was shaken at intervals during the incubation, finally centrifuged and the supernatant used as extract.

The tumour cell extract presumably contained ample essential solutes not sufficiently provided in the unmodified basic medium. The practical limitation of the latter set by the necessity of using a relatively high initial population of tumour cells to obtain adequate multiplication and the consequent depletion of the medium within 24 hours was overcome by the inclusion of the tumour cell extract in the basic medium. Sarcoma 37 tumour cells grew in this supplemented medium for several days when initial population densities 1/100th of that of the native ascitic fluid were used. Ehrlich carcinoma ascites tumour cells behaved similarly in supplemented homologous media in which the soluble factors were supplied by the cell extract.

DISCUSSION

The media described were able to support the growth *in vitro* of the ascites tumour cells tested. They had the disadvantage that two of the components were prepared from native ascites tumour fluid. No suitable substitutes for ascitic plasma and serum or tumour cell extract were found. The latter was not entirely replaceable by embryo extract.

Ehrlich carcinoma and Sarcoma 37 ascites tumour cells differed quantitatively in their dependence on the concentration in the culture medium of the soluble essential substances released by the cells and supplied in the cell extract. The ascitic plasma used presumably contained a low proportion of these substances since Ehrlich carcinoma cells grew at certain population densities without the addition of cell extract. The importance of these soluble factors for the viability and growth of ascites tumour cells has been discussed previously (Powell, 1957). The present researches confirmed this earlier work. The limiting factors in the dependence of the tumour cells upon these substances may be the ratios between the rates at which they are synthesised and exchanged between the cells and medium. The tumour cell extract component of the media may be more important for the growth of the tumour cells than the plasma fraction.

The media described would appear to be suitable for quantitative studies on the effects of cytotoxic agents on tumour cell populations under controlled conditions *in vitro*. Relatively large initial populations of tumour cells, 1/8th of the *in vivo* density, could be studied for periods of 24 hours or smaller initial populations for longer periods. Cultivated tumour cells inoculated intra-peritoneally into mice gave rise to normal ascites tumours.

The roles of the enzymes used in the basic medium are uncertain. Trypsin has been used frequently to dissociate the cells of tissues; Moscona (1952), and Moscona and Moscona (1952) used it upon chick embryo tissues. Willmer (1945, 1954) has described its use for this purpose and also its effect in causing fibrocytes to round up with retention of viability. This latter property may be associated with its beneficial effects in the medium. On the other hand, it is improbable that the enzyme remained active for long periods in the presence of the considerable amounts of protein in the medium. It is possible that its proteolytic effect upon the ascitic plasma was important since carcinoma cells liberate proteolytic enzymes and the ascites cells were cultivated at densities of population lower than those *in vivo*. The enzyme perhaps compensated for the reduced numbers of cells in this respect and may have liberated nutrients form the plasma proteins.

Hyaluronidase, which depolymerises hyaluronic acid derivatives, reduces cartilaginous matrix *in vitro* (Paff and Seifter, 1950) and dissolves intercellular cementing substance. Possibly it affected the surfaces of the ascites cells. Embryo extract presumably functioned in virtue of its content of growth substances.

During the past decade many reports of successful cultivation of cell suspended in fluid media have been made (Paul, 1959). These include L strain fibroblasts (Earle *et al.*, 1954, 1956 ; Danes, 1957 ; McLimans *et al.*, 1957), HeLa human carcinoma cells (Gey, Bang and Gey, 1954; Earle *et al.*, 1956). In most of these instances the suspended cells have been subjected to violent continuous agitation and standard media have been used. A substrain of de Bruyn's MB lymphoblast has been grown in slow suspension by Owens, Gey and Gey (1953) but these cells are atypical and altered from the parent strain.

The present researches differ from these examples of true suspension cultures in that the cells lay mainly on the surfaces of the culture vessels although smoothly contoured dividing cells were found free in the medium and suspensions of cells were easily recovered from the roller-tubes. As with the lymphoblast cells of de Bruyn the present results were probably due to the innate properties of the ascites tumour cells reinforced by a medium consisting largely of their normal pabulum.

SUMMARY

A fluid culture medium which supports the growth of Ehrlich carcinoma ascites tumour cells *in vitro* is described.

This medium is suitable for short term assays of the effects of cytotoxic agents on the cultivated tumour cells, since they can be quantitatively recovered for enumeration.

The numbers of cells inoculated into this medium must lie within narrow limits for successful cultivation.

Supplementation of the basic medium with a saline extract of homologous ascites tumour cells permits successful cultivation with much smaller initial numbers of cells. Sarcoma 37 ascites tumour cells, which fail to grow in the basic medium, multiply in supplemented medium.

This saline extract is presumed to contain soluble protective factors which diffuse from the cells into the medium. The concentration of these substances determines the number of cells which may be successfully inoculated in cultures.

I am indebted for assistance with the *in vitro* researches to Mr. G. A. Butcher and with the maintenance of the tumours *in vivo* to Mr. F. Butcher.

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REFERENCES

British Empire Cancer Campaign.—(1951) *Ann. Rep.*, **29**, 57.

Danes, B. S.—(1957) *Exp. Cell. Res.*, **12**, 169.

Earle, W. R.—(1943) *J. nat. Cancer Inst.*, **4**, 165.

Idem, Bryant, J. C., Schilling, E. L. and Evans, V. J.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 666.

Idem, Schilling, E. L., Bryant, J. C. and Evans, V. J.—(1954) *J. nat. Cancer Inst.*, **14**, 1159.

Gey, G. O., Bang, F. B. and Gey, M. K.—(1954) *Tex. Rep. Biol. Med.*, **12**, 805.

Heilbrunn, L. Y. and Wilson, W. L.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **70**, 179.

Klein, G.—(1950) *Cancer*, **3**, 1052.—(1951) 'The Production of Ascites Tumours in Mice and Their Use in Studies on Some Biological and Chemical Characteristics of Neoplastic Cells'. Uppsala (Almqvist and Wicksells Botryckeri).

Idem and Révész, L.—(1953) *J. nat. Cancer Inst.*, **14**, 229.

Lettré, H.—(1941) *Z. physiol. Chem.*, **268**, 59; **271**, 192.—(1950) *Z. Krebsforsch.*, **57**, 1.

McLimans, W. F., Giardinello, F. E., Davis, E. V., Kucera, C. J. and Rake, G. W. —(1957) *J. Bact.*, **74**, 768.

Moscona, A.—(1952) *Exp. Cell Res.* **3**, 535.

Moscona, H. and Moscona, A.—(1952) *J. Anat.*, **86**, 287.

Owens, O. von H., Gey, G. O. and Gey, M. K.—(1953) *Proc. Amer. Ass. Cancer Res.*, **1**, 41.

Paff, G. H. and Seifter, J.—(1950) *Anat. Rec.*, **106**, 525.

Paul, J.—(1959) 'Cell and Tissue Culture'. London (Livingston).

Powell, A. K.—(1957) *Brit. J. Cancer*, **11**, 274, 280, 478.

Schrek, R.—(1936) *Amer. J. Cancer*, **28**, 389.

Warburg, O. and Heipler, E.—(1952) *Z. Naturf.*, 7b, 193.

Willmer, E. N.—(1945) 'Growth and Form in Tissue Cultures'. *In* 'Growth and Form Essays presented to D'Arcy Thompson', ed. W. Le Gros Clark and P. B. Medawar. Oxford (University Press).—(1954) 'Tissue Culture', 2nd edition. London (Methuen).

URINARY β-GLUCURONIDASE ACTIVITY IN CANCER OF THE BLADDER AND OTHER DISEASES

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A THEORY of the mode of action of aromatic amines in producing carcinoma of the bladder in man has been developed by Boyland and his colleagues (Boyland, Wallace and Williams, 1955; Allen, Boyland, Dukes, Horning and Watson, 1957; Wallace, 1959). They pointed out that in men working in the chemical industry who have had contact with α- or β-naphthylamine or benzidine the incidence of cancer of the bladder is very high, but other organs are not affected. They suggest that the absorbed amines are carried to the liver where they are metabolised to *ortho*-aminophenols which are very rapidly conjugated with sulphate or glucuronic acid by similar mechanisms to those they have demonstrated in rat liver (Booth, Boyland and Manson, 1955) and that these conjugates are eventually excreted by the kidney. In the urine the glucosiduronic acids (but not sulphates which are resistant to hydrolysis) are exposed to the action of hydrolytic enzymes and thus free *ortho*-aminophenols are liberated. It is known that several of these *ortho*-aminophenols, including 2-amino-1-hydroxynaphthalene can induce bladder cancer in mice and dogs (Hueper and Wolfe, 1937; Hueper, Wiley and Wolfe, 1938; Bonser, Bradshaw, Clayson and Jull, 1956; Allen *et al.*, 1957; and Wallace, 1959).

In people not exposed to the chemical hazard it is suggested that *ortho*-aminophenols derived from the metabolism of tryptophan may be one of the causative factors in bladder cancer (Allen *et al.*, 1957).

In a part of a series of investigations undertaken to examine this hypothesis concerning the induction of bladder cancer, Boyland *et al.* (1955) demonstrated that β-glucuronidase activity is almost always increased in the urine of patients with cancer of the bladder. They suggested that investigation of urinary β-glucuronidase activity might be of value in the prognosis of such patients.

Because of the concentration of suitable cases available to the Bristol Bladder Tumour Registry, it was decided to investigate the significance of this urinary enzyme, employing a wide range of controls.

MATERIAL AND METHODS

Material

The control series fell into three main groups:

Group 1.—This group consisted of 32 healthy subjects aged 17–61 years with an average age of 33 years. Twenty-three (72 per cent) were male.

Group 2.—There were 87 patients in this group with a wide range of genito-urinary (g.u.) diseases other than tumours. The average age was 44 years with a range of 14–79 years.

Group 3.—Group 3 was a "miscellaneous" group of 120 patients with an average age of 51 years and a range of 16–82 years, with various diseases not connected with the genito-urinary tract. Patients with bone fractures were not included but were assessed separately since they were found to have a significantly raised enzyme activity.

As the patients in Groups 2 and 3 were selected at random from the hospital, the average age was lower than in the patients with cancer of the bladder. Two sub-groups consisting of patients over 50 years of age were therefore isolated from these main groups.

In 2a, the sub-group of the other genito-urinary diseases, the average age was 59 years with 19 (91 per cent) male. In 3a, the sub-group of the "miscellaneous" diseases, the average age was 60 years, of which 16 (47 per cent) were male.

Group 4.—Group 4 consisted of bladder carcinoma patients who came into hospital mainly for review cytoscopy. There were 86 patients with an average age of 63 years ranging from 38–81 years and of these 82 per cent were male. In none of these was there any evidence of association with the dye industry.

Method

Twenty-four hour specimens of urine were collected; the urine preservative for about three-quarters of the investigation was 10 ml. benzene, but in the later stages, in accordance with the revised method of Boyland *et al.* (1957), this was changed to 10 ml. of a 20 per cent solution of thymol in benzene. The thymol with benzene had no effect on the enzyme level but had a greater bacteriocidal effect than benzene alone. It was found that the enzyme in urine of normal pH was stable for at least seven days with the preservative at room temperature; this was in agreement with the results of Boyland *et al.* (1955). In the bladder cancer group the urine was collected before cystoscopy. In some patients it was unavoidable that pre-operative drugs were given during the last hour or so of the 24-hour urine collection. In all groups it was noted whether the patient had been receiving drugs or had been pyrexial during the collection and whether there had been any operative procedure within the previous fortnight. The pH and specific gravity of the urine were determined. In some cases the creatinine value was estimated as a further check on whether the collection had been complete. Of the 101 specimens on which such determinations were made only eight had a creatinine value of less than 0·5 g./24 hours. This was taken as an indication of the level of accuracy in the urine collection. Unfortunately these estimations were not carried out at the beginning of the whole investigation where collection errors might be expected to have been greater.

Approximately 15 ml. of urine was centrifuged in a conical tube at 2000 r.p.m. for at least 10 minutes. In a proportion of cases one drop of the deposit was examined under the microscope and patients with deposits containing more than 20 red blood corpuscles in the drop were considered to have excess red cells. Red cells have negligible β-glucuronidase activity (Fishman, Springer and Brunetti, 1948), but their presence in urine indicates the possibility of contamination with serum which has an activity of up to three times that of urine in normal subjects.

The β-glucuronidase activity of the supernatant fluid, and in some cases the urinary deposit also, was determined by a slight modification of the method of Boyland, Gasson and Williams (1957) which was derived from the original method of Talalay, Fishman and Huggins (1946). Duplicate samples of either 1 ml. of

supernatant urine or an appropriate volume of the deposit resuspended in distilled water were incubated with 1 ml. acetate buffer (0·2 M, pH 4·5) and 1 ml. phenolphthalein mono-β-glucuronic acid (0·05 per cent in 10 per cent ethanol) in stoppered tubes at 37° C. for 18 hours. At the end of this time 1 ml. of urine was added to the blank and a sufficient volume of 0·1 N NaOH was added to each tube just to reach the pink coloration of the free phenolphthalein end-point. Then 1 ml. of glycine buffer (0·4 M, pH 10·5) was added. The solutions were centrifuged for 5 minutes at 2000 r.p.m. and the absorption at 550 mμ read in a Hilger spectrophotometer. With urines of different buffering power it was necessary to add different volumes of 0·1 N NaOH to reach the end-point and allowance was made for the different final volumes obtained. The activity was expressed in the same unit used by Boyland *et al.* (1957), namely, 1 unit is the amount of enzyme liberating 1 μg. of phenolphthalein per hour at 37° C.

In 50 individuals the urine was investigated to determine the presence of heat stable β-glucuronidase inhibitors or activators. The enzyme activity of various concentrations of urine diluted with distilled water was compared with the same concentrations of urine diluted with boiled urine. The results were plotted graphically, the percentage activity against the amount of dilution. In those cases where the urine diluted with water gave a curve on the graph, while the same urine diluted with boiled urine gave a straight line, it was assumed that an inhibitor was present. Since these investigations were made on the unpurified enzyme the term inhibitor was used in its broadest sense. This inhibitor decreased in concentration as the urine was diluted with distilled water but remained constant when diluted with boiled urine (Boyland *et al.*, 1955). The inhibitor index (I^{50}) was calculated as the ratio:

$$\frac{\text{Unit of activity of 1 ml of a solution of urine diluted with an equal volume of distilled water}}{\text{Unit of activity of 1 ml. of a solution of urine diluted with an equal volume of boiled urine}}.$$

With regard to the reproducibility of the method, the enzyme activity in 12 equal aliquots of urine was estimated ; the mean value of the liberated phenolphthalein was 4·9 μg./ml. with a standard deviation of 0·1 representing an error of almost 3 per cent.

The accuracy of the method was more difficult to determine. The chief errors arose from the different ionic and non-ionic constituents and buffering powers of the different urines. As Boyland *et al.* (1957) have shown, 0·2 M acetate buffer was sufficient to bring any urine within the normal range to a pH of 4·5–4·8. In the investigation of urines with pH over 7·0 it was necessary to adjust the urine to pH 4·5 with acetic acid before adding the pH 4·5 buffer. At the end of the experiment, after the addition of 0·1 N NaOH and 0·4 M glycine, it was found that the pH ranged from 10·1–10·5. Since the standard phenolphthalein curve was determined at pH 10·5 there was, in some determinations, an error due to a difference of the phenolphthalein colour intensity at a pH different from that of the standard. The magnitude of this error was determined in a series of 61 urines. The usual duplicate solutions were set up for incubation with an extra tube containing 1 ml. urine, 1 ml. 0·2 M acetate buffer and 0·5 ml. 10 per cent ethanol. At the end of the incubation time the same volumes of 0·1 N NaOH and 0·4 M glycine were added to the extra tube as were to the duplicate tubes and 0·5 ml.

of a standard solution of phenolphthalein was then added to this third tube. The colour developed in this tube was compared with the colour developed by the standard phenolphthalein in glycine buffer at pH 10·5. The percentage difference was calculated. The mean percentage difference for the 61 urines was 6·9 with an S.D. of ±4·5. This difference included the error due to the different final pH and the small salt and protein indicator error of phenolphthalein.

RESULTS

Before dealing with the range of β-glucuronidase activity in the cases of cancer of the bladder and the different groups of controls, the results of investigating various factors which might be expected to influence this enzyme activity are presented. A wide range of drugs was given to the patients as part of their treatment. Excluding the steroid hormones, particularly cortisone, which appeared to raise the enzyme activity, the general picture was that most drugs and antibiotics did not have any appreciable effect. As Boyland and Williams (1956) had noted, patients with pyrexia often had a raised enzyme value.

TABLE I.—*β-glucuronidase Activity of the Urinary Sediment*

(*a*) In normal subjects

Initials	Age	Sex	β-glucuronidase activity			
			Sediment		Supernatant	
			Units/ml. original urine	Units/day	Units/ml. urine	Units/day
G. R—	35	M.	0	0	0·36	765
P. K—	34	,,	0·03	32	0·76	830
K. C—	24	,,	0·04	56	1·37	1800
L. B—	60	,,	0·04	83	0·83	1910
M. T—	19	,,	0·07	76	1·02	1140
J. K—	25	F.	0·07	85	1·3	1610
J. R—	29	,,	0·07	95	1·07	1400
M. B—	20	M.	0·09	102	0·83	963
C. P—	30	F.	0·10	150	0·73	1300
D. F—	25	M.	0·10	166	0·69	1120
M. J—	29	F.	0·11	176	1·0	1650
F. K—	44	M.	0·12	60	1·02	1150
D. L—	18	F.	0·12	76	1·15	697
M. B—	28	M.	0·13	203	1·05	1570
A. K—	20	F.	0·14	199	0·51	724
V. B—	20	,,	0·17	142	0·94	795
P. V—	28	,,	0·18	435	0·87	2100
J. A—	21	,,	0·31	252	0·96	784

(a) *β-glucuronidase activity in urinary sediment*

Table I (*a*) demonstrates that the β-glucuronidase activity of the small urinary deposits found in 18 normal subjects ranged from 0–450 units per day and the deposit in women usually had a higher activity than in men. The enzyme level was increased in some cases of bladder cancer (Table I (*b*)) and also in other diseases. During the investigation of 64 specimens there appeared to be no consistent relation between the activity in the deposit and the activity of the supernatant urine.

TABLE I.—*β-glucuronidase Activity of the Urinary Sediment*

(*b*) In patients with cancer of the bladder

Patient No.	Age	Sex	β-glucuronidase activity: Sediment Units/ml. original urine	Sediment Units/day	Supernatant Units/ml. urine	Supernatant Units/day	
165	76	M.	0	0	0·48	1140	−
405	78	,,	0	0	0·66	920	+
45	72	,,	0·01	34	1·7	3900	+
404	65	,,	0·014	27	1·5	2860	−
377	59	,,	0·05	30	1·74	1060	+
196	68	F.	0·05	132	0·88	2340	−
166	72	M.	0·1	194	0·49	930	+
243	70	,,	0·23	426	0·85	1560	−
154	62	,,	0·37	985	1·1	3100	+
		With red corpuscles contamination					
164	66	F.	1·05	1439	1·77	2420	+
16	55	M.	1·36	2860	1·02	2140	+

+ = A tumour recurrence was found during the cystoscopy immediately following the urine collection.
− = No recurrence.

(b) *The presence of β-glucuronidase inhibitors in the supernatant urine* (Table II, Fig. 1)

Of 52 men investigated from all groups, 45 (86 per cent) had an I^{50} of more than 1·1 which was taken to indicate the presence of inhibitors. In 21 women,

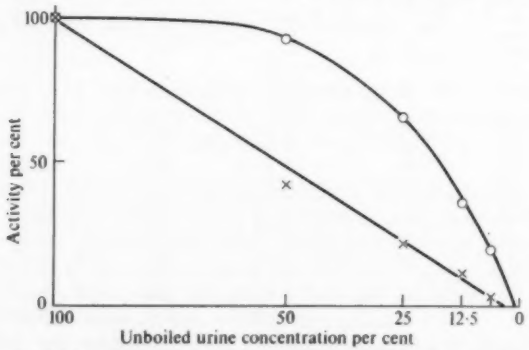

FIG. 1.—Inhibition of urinary β-glucuronidase by substances present in urine.
○ Diluted with distilled water.
× Diluted with boiled urine.

11 (52 per cent) had an I^{50} value of more than 1·1. If these results were due to the presence of a single substance, this inhibitor was present in men as frequently in cancer of the bladder (in 12 out of 13 cases) as in other disease (28 out of 34) or in normal subjects (in each of the 5 subjects investigated). In the urine from

women the corresponding results were : cancer of the bladder (one out of two cases), other disease (7 out of 12) and normal subjects (3 out of 7).

TABLE II.—*The Inhibitor Index of Supernatant Urine*

Group	Patient's No. or initials	Sex	I^{30}
1. Normal subjects	C. P—	F.	0·42
	A. K—	„	1·00
	M. C—	„	1·00
	J. G—	„	1·10
	J. H—	„	1·30
	J. C—	„	1·40
	M. B—	M.	1·47
	D. F—	„	1·50
	G. R—	„	1·56
	J. B—	„	1·70
	P. V—	F.	1·80
	F. K—	M.	1·90
2. Other genito-urinary diseases	241	F.	0·03
	371	M.	1·00
	299	F.	1·00
	139	M.	1·00
	249	F.	1·06
	251	M.	1·10
	259	„	1·17
	250	„	1·17
	255	„	1·18
	220	„	1·20
	237	„	1·22
	138	„	1·30
	256	„	1·30
	—	„	1·30
	257	„	1·36
	231	„	1·41
	121	„	1·43
	422	„	1·44
	High urinary pH		
	227	F.	1·20
	198	M.	1·20
	140	„	1·30
	112	„	1·44
	52	„	1·54
	Within 14 days after operation		
	205	F.	0·80
	240	„	1·08
	254	M.	1·18
	135	„	1·60
	248	„	2·25
3. "Miscellaneous" diseases	15	F.	0·76
	238	M.	1·10
	193	„	1·10
	423	„	1·17
	223	„	1·36
	242	„	1·45
	224	„	1·60
	228	„	1·60
	195	„	2·00
	219	„	2·43
	13	F.	2·63
	High urinary pH		
	136	M.	2·20
	Within 14 days after operation		
	245	M.	1·05
	247	„	1·17
	204	„	1·66
	199	„	1·73
	202	„	2·00
	203	„	2·0
4. Cancer of the bladder	196	F.	0·79
	165	M.	1·00
	154	„	1·17
	45	„	1·18
	405	„	1·27
	166	„	1·43
	152	„	1·48
	404	„	1·50
	279	„	1·55
	377	„	1·57
	16	„	1·70
	378	„	1·78
	With red cell contamination		
	164	F.	1·80
	16	M.	2·59

The urine from 3 women contained an "activator" of some kind, since on dilution with distilled water the decrease in enzyme activity was greater than on dilution with boiled urine (Fig. 2). In each of 4 normal subjects and 2 patients on whom these investigations have been repeated, the inhibition or activation originally demonstrated remained unchanged.

(c) *Urinary β-glucuronidase activity during the menstrual cycle*

An attempt was made to correlate the enzyme activity with the phases in the normal menstrual cycle. Four normal women between the ages of 20–30 years

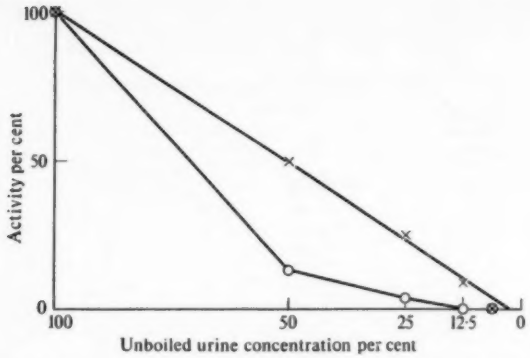

FIG. 2.—Activation of urinary β-glucuronidase by substances present in some women's urine.

○ Diluted with distilled water.
× Diluted with boiled urine.

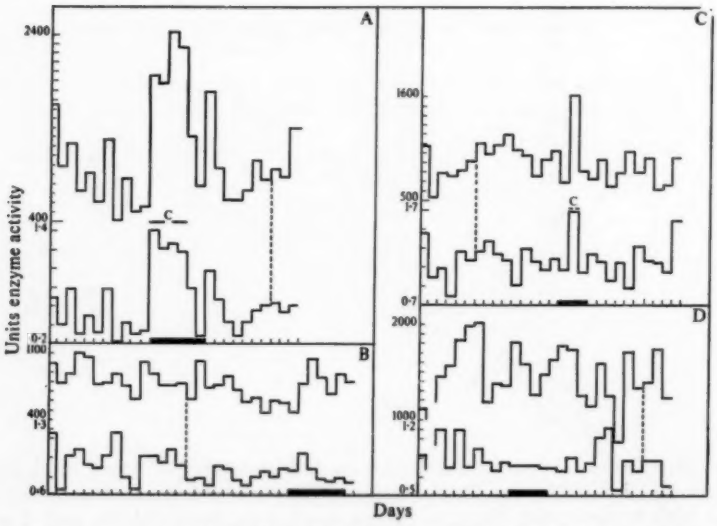

FIG. 3.—Urinary enzyme activity throughout the menstrual cycle in four young women. For each individual the lower graph represents the activity per ml. urine and the upper graph represents the total daily enzyme output.

Menstruation.
Contaminated by menstrual blood.
Time of ovulation.

collected 24-hour specimens throughout one cycle and noted the time of ovulation by recording waking temperatures. Since the cycles were normal it was assumed that peak oestrogen output occurred at about ovulation time and just before menstruation (Pedersen-Bjergaard and Tønnesen, 1948). The urinary β-glucuronidase activity appeared to be unrelated to the phases of the menstrual cycle except that in specimens grossly contaminated with menstrual blood and tissue the activity was elevated (Fig. 3).

TABLE III.—*The β-glucuronidase Activity in Infected Urine where the pH is Within Normal Limits*

Patient No.	pH	β-glucuronidase activity (units/ml.)	Organism
299	5·2	1·10	Tubercle bacillus.
174	5·2	1·77	Mixed coliforms.
148	5·3	0·21	Coliforms and *B. proteus*.
150	5·6	0·66	Coliforms.
122	5·6	0·84	Coliforms and staphylococci.
109	5·6	1·50	*B. proteus*.
112	5·7	0·96	*E. coli*.
244	6·0	0·99	Mixed coliforms.
130	6·0	1·29	*E. coli*.
110	6·0	1·51	*Ps. pyocyanea*.
161	6·0	1·51	*Ps. pyocyanea*.
230	6·0	1·51	*E. coli*.
79	6·0	1·70	*E. coli*.
194	6·2	0·3	*E. coli*.
240	6·2	0·63	*E. coli*.
83	6·2	0·93	Mixed with *E. coli*.
256	6·2	1·05	*Aerobacter aerogenes*. *Ps. pyocyanea*.
120	6·2	1·08	*Aerobacter aerogenes*.
242	6·2	1·11	Mixed coliforms.
218	6·3	1·10	Mixed coliforms and *Staphylococcus aureus*.
274	6·4	0·51	*E. coli*.
58	6·4	0·66	Mixed.
253	6·4	0·90	*E. coli*.
226	6·4	1·41	*Aerobacter aerogenes*.
301	6·58	0·90	*E. coli*.
246	6·6	1·60	Coliforms.
250	6·8	0·81	Coliforms and Enterococci.

(d) *The effect of alkaline conditions of the urine upon the β-glucuronidase activity* (Tables III and IV)

With slight urinary infection where the pH was not raised above pH 7·0 the enzyme activity was not significantly altered (Table III). In 5 patients with cancer of the bladder the urine was grossly infected and in 4 cases the pH was raised to a value of 9·0 or more. In these patients the urinary enzyme activity was increased. In patients suffering from other diseases where the pH of the urine was abnormally high, in several cases (6 out of 20), the enzyme activity was elevated (Table IV). The effect of incubating normal urine with *B. proteus* or *E. coli* before the assay was variable, the enzyme activity being sometimes increased and sometimes decreased. When the pH of normal urine was increased by the addition of alkali the enzyme activity fell.

TABLE IV.—*Urinary β-glucuronidase Activity in Patients with Urine of High pH*

Disease	Patient No.	pH	β-glucuronidase activity: Units/ml. urine	β-glucuronidase activity: Units/day	Organism
Cancer of the bladder	296	7·14	4·95	3,960	—
	287	9·0	2·17	4,180	Enterococci.
	213	9·0	2·40	3,820	—
	278	9·2	2·77	11,400	*B. proteus.*
	305	>9·0	3·28	3,180	—
Any other disease	222	7·2	0·93	1,311	*B. proteus.*
	169	7·28	0·69	2,640	Sterile.
	86	7·4	1·14	2,040	Sterile.
	81	7·5	2·30	1,840	—
	63	7·6	1·70	3,570	*Aerobacter aerogenes.*
	160	8·0	2·19	4,420	Non-haemolytic streptococci.
	295	>8·0	0	0	Coliforms + *Streptococcus faecalis.*
	108	>8·0	0·81	2,090	Mixed coliforms.
	50	>8·0	1·18	2,840	Coliform.
	227	>8·0	2·04	4,896	—
	140	8·5	0·72	453	*E. coli.*
	105	9·0	1·68	2,140	*B. proteus.*
	198	9·0	7·65	33,600	*Streptococcus faecalis* and *B. proteus.*
	64	>9·0	0·72	630	Diphtheroid.
	248	>9·0	1·14	1,530	*Aerobacter aerogenes.*
	26	>9·0	1·57	2,155	*B. proteus.*
	89	>9·0	2·93	1,570	*B. proteus.*
	149	>9·0	3·96	10,300	—
	54	>9·0	4·20	11,050	—
	112	>9·0	9·45	5,760	—

(e) *Urinary β-glucuronidase activity after operation* (Table V)

It was found that within 8 days after major operations performed on parts of the alimentary canal the enzyme activity was significantly increased, which is in agreement with the observation of Boyland and Williams (1956). On the other hand minor operations upon skin or fairly superficial muscle did not result in an elevation of the enzyme activity. The effect of operations on the genito-urinary tract upon the enzyme activity was variable.

TABLE V.—*Urinary β-glucuronidase Activity within 8 Days of Operation*

Type of surgery	n	Urinary β-glucuronidase activity: Units/ml. urine: Mean	S.D.	S.E.	"t" test against normal subjects: t	P	Units/day: Mean	S.D.	S.E.	"t" test against normal subjects: t	P
Minor	20	1·19	0·39	0·088	0·98	Between 0·4 and 0·3	1523	569	127	0·52	0·6
Major	13	3·72	1·68	0·466	8·08	Less than 0·01*	3534	1750	486	5·37	Less than 0·01*

S.E. = Standard error of the mean.
* Significantly different.

β-glucuronidase activity in normal subjects, in cancer of the bladder and in other diseases (Table VI)

Group 1.—In normal subjects the mean activity/ml. urine was 1·05 units with a standard error of the mean of 0·098 and the mean daily excretion was 1405 units/day with a standard error of 160. Boyland *et al.* (1957) found the normal range to be 0·05–1·2 units/ml. and when the daily outputs were calculated from their published figures the mean was 1304 units/day with a range of 162–4890 units.

TABLE VI.—*Urinary β-glucuronidase Activity in Normal Subjects and in Patients with Different Diseases*

Group	Average age (years)	Units/ml.			"*t*" test against normal subjects		Units/day			"*t*" test against normal subjects	
		n	Mean	S.E.	S.D.	*t*	*P*	Mean	S.E.	S.D.	*t*	*P*
1. Normal subjects	33	32	1·05	0·098	0·55	—	—	1405	160	893	—	—
2. G.U. diseases (total age range)	44	41	1·21	0·122	0·78	0·98	Between 0·4 and 0·3	2353	270	1707	2·8	0·01*
2a. G.U. diseases (over 50 years of age)	59	21	1·31	0·227	1·04	1·14	Between 0·3 and 0·2	2251	441	2023	2·06	Between 0·05 and 0·02*.
3. "Miscellaneous" diseases (total age range)	51	55	1·16	0·084	0·62	0·82	Between 0·5 and 0·4	1719	141	1047	1·4	Between 0·2 and 0·1.
3a. "Miscellaneous" diseases (over 50 years of age)	60	34	1·35	0·121	0·70	1·91	Between 0·1 and 0·05	1649	163	950	1·02	Between 0·3 and 0·2.
3b. Fractures	43	27	2·55	0·213	1·24	6·14	0·01*	2990	321	1697	4·55	Less then 0·001*.
4. Cancer of the bladder	63	71	1·18	0·068	0·58	1·073	Between 0·3 and 0·2	1831	100	831	2·3	0·025*

S.E. = Standard error of the mean.
* Significantly different.

TABLE VII.—*Daily Volume of Urine Excreted by Normal Subjects and Patients with Different Diseases*

Group	Volume of urine excreted (ml.)			"*t*" test against normal subjects	
	Mean	S.E.	S.D.	*t*	*P*
1. Normal subjects	1419	110	577	—	—
2. Genito-urinary diseases (total age range)	2094	137	901	3·52	Less than 0·001*.
2a. Genito-urinary diseases (over 50 years of age)	1824	165	755	2·108	Between 0·05 and 0·02*.
3. "Miscellaneous" diseases (total age range)	1462	87	635	0·356	Between 0·8 and 0·7.
3a. "Miscellaneous" diseases (over 50 years of age)	1360	34	625	0·379	Between 0·8 and 0·7.
3b. Fractures	1173	108	571	1·529	Between 0·2 and 0·1.
4. Cancer of the bladder	1739	68	601	2·408	Between 0·02 and 0·01*.

* Significantly different.

TABLE IV.—*Urinary β-glucuronidase Activity in Patients with Urine of High pH*

Disease	Patient No.	pH	β-glucuronidase activity: Units/ml. urine	β-glucuronidase activity: Units/day	Organism
Cancer of the bladder	296	7·14	4·95	3,960	—
	287	9·0	2·17	4,180	Enterococci.
	213	9·0	2·40	3,820	—
	278	9·2	2·77	11,400	*B. proteus.*
	305	>9·0	3·28	3,180	—
Any other disease	222	7·2	0·93	1,311	*B. proteus.*
	169	7·28	0·69	2,640	Sterile.
	86	7·4	1·14	2,040	Sterile.
	81	7·5	2·30	1,840	—
	63	7·6	1·70	3,570	*Aerobacter aerogenes.*
	160	8·0	2·19	4,420	Non-haemolytic streptococci.
	295	>8·0	0	0	Coliforms + *Streptococcus faecalis.*
	108	>8·0	0·81	2,090	Mixed coliforms.
	50	>8·0	1·18	2,840	Coliform.
	227	>8·0	2·04	4,896	—
	140	8·5	0·72	453	*E. coli.*
	105	9·0	1·68	2,140	*B. proteus.*
	198	9·0	7·65	33,600	*Streptococcus faecalis* and *B. proteus.*
	64	>9·0	0·72	630	Diphtheroid.
	248	>9·0	1·14	1,530	*Aerobacter aerogenes.*
	26	>9·0	1·57	2,155	*B. proteus.*
	89	>9·0	2·93	1,570	*B. proteus.*
	149	>9·0	3·96	10,300	—
	54	>9·0	4·20	11,050	—
	112	>9·0	9·45	5,760	—

(e) *Urinary β-glucuronidase activity after operation* (Table V)

It was found that within 8 days after major operations performed on parts of the alimentary canal the enzyme activity was significantly increased, which is in agreement with the observation of Boyland and Williams (1956). On the other hand minor operations upon skin or fairly superficial muscle did not result in an elevation of the enzyme activity. The effect of operations on the genito-urinary tract upon the enzyme activity was variable.

TABLE V.—*Urinary β-glucuronidase Activity within 8 Days of Operation*

Type of surgery	n	Urinary β-glucuronidase activity: Units/ml. urine: Mean	S.D.	S.E.	"t" test against normal subjects: t	P	Units/day: Mean	S.D.	S.E.	"t" test against normal subjects: t	P
Minor	20	1·19	0·39	0·088	0·98	Between 0·4 and 0·3	1523	569	127	0·52	0·6
Major	13	3·72	1·68	0·466	8·08	Less than 0·01*	3534	1750	486	5·37	Less than 0·01*

S.E. = Standard error of the mean.
* Significantly different.

β-glucuronidase activity in normal subjects, in cancer of the bladder and in other diseases (Table VI)

Group 1.—In normal subjects the mean activity/ml. urine was 1·05 units with a standard error of the mean of 0·098 and the mean daily excretion was 1405 units/day with a standard error of 160. Boyland *et al.* (1957) found the normal range to be 0·05–1·2 units/ml. and when the daily outputs were calculated from their published figures the mean was 1304 units/day with a range of 162–4890 units.

TABLE VI.—*Urinary β-glucuronidase Activity in Normal Subjects and in Patients with Different Diseases*

Group	Average age (years)	Units/ml. n	Mean	S.E.	S.D.	"t" test against normal subjects t	P	Units/day Mean	S.E.	S.D.	"t" test against normal subjects t	P
1. Normal subjects	33	32	1·05	0·098	0·55	—	—	1405	160	893	—	—
2. G.U. diseases (total age range)	44	41	1·21	0·122	0·78	0·98	Between 0·4 and 0·3	2353	270	1707	2·8	0·01*
2a. G.U. diseases (over 50 years of age)	59	21	1·31	0·227	1·04	1·14	Between 0·3 and 0·2	2251	441	2023	2·06	Between 0·05 and 0·02*.
3. "Miscellaneous" diseases (total age range)	51	55	1·16	0·084	0·62	0·82	Between 0·5 and 0·4	1719	141	1047	1·4	Between 0·2 and 0·1.
3a. "Miscellaneous" diseases (over 50 years of age)	60	34	1·35	0·121	0·70	1·91	Between 0·1 and 0·05	1649	163	950	1·02	Between 0·3 and 0·2.
3b. Fractures	43	27	2·55	0·213	1·24	6·14	0·01*	2990	321	1697	4·55	Less then 0·001*.
4. Cancer of the bladder	63	71	1·18	0·068	0·58	1·073	Between 0·3 and 0·2	1831	100	831	2·3	0·025*

S.E. = Standard error of the mean.
* Significantly different.

TABLE VII.—*Daily Volume of Urine Excreted by Normal Subjects and Patients with Different Diseases*

Group	Volume of urine excreted (ml.) Mean	S.E.	S.D.	"t" test against normal subjects t	P
1. Normal subjects	1419	110	577	—	—
2. Genito-urinary diseases (total age range)	2094	137	901	3·52	Less than 0·001*.
2a. Genito-urinary diseases (over 50 years of age)	1824	165	755	2·108	Between 0·05 and 0·02*.
3. "Miscellaneous" diseases (total age range)	1462	87	635	0·356	Between 0·8 and 0·7.
3a. "Miscellaneous" diseases (over 50 years of age)	1360	34	625	0·379	Between 0·8 and 0·7.
3b. Fractures	1173	108	571	1·529	Between 0·2 and 0·1.
4. Cancer of the bladder	1739	68	601	2·408	Between 0·02 and 0·01*.

* Significantly different.

TABLE VIII

(*a*) Comparison of the 24-hour enzyme activity in subjects from the normal and " miscellaneous " groups who had excreted over 1700 ml. urine, with the activity in patients with carcinoma of the bladder and with other genito-urinary diseases.

				" *t* " tests							
				Urine volumes				24-hour enzyme activity			
		Mean volume urine excreted	Mean daily enzyme activity	Against carcinoma bladder group		Against other g.u. diseases		Against carcinoma bladder group		Against other g.u. diseases	
Group	n	(ml.)	(units)	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*
1+3>1700	19	2150	2355	2·78	<0·01*	0·253	0·80	2·37	0·02*	0·005	>0·9

(*b*) Comparison of the 24-hour enzyme activity in patients with carcinoma of the bladder and other genito-urinary diseases who had excreted less than 1900 ml. urine, with the activity in normal subjects.

				" *t* " test against normal subjects			
				Urine volumes		24-hour enzyme activity	
		Mean volume urine excreted	Mean enzyme activity				
Group	n	(ml.)	(units)	*t*	*P*	*t*	*P*
2+4<1900	55	1323	1687	0·899	Between 0·4 and 0·3	1·453	Between 0·2 and 0·1

* Significantly different.

Groups 2, 3 *and* 4.—The results in this section are from urine within the normal pH range and not contaminated by excess red cells. During the urine collection the patients were not suffering from pyrexia, had not undergone operations within the previous fortnight and were not having steroid hormone therapy.

In the original " miscellaneous " group it became obvious that patients with fractures had an enzyme activity higher than any other. These cases were therefore removed and considered separately.

Apart from this (Group 3b) the β-glucuronidase activity per ml. urine in any of the groups (2, 2a, 3, 3a, or 4) was not significantly different from that in normal subjects.

The daily enzyme output in both the total " miscellaneous " group (3) and its sub-group of patients over 50 years of age was also not significantly different from the normal value. The enzyme output per day, however, in the total " other genito-urinary diseases "(Group 2) and in its sub-group was significantly increased as it was also in the group of cancer of the bladder cases (4).

It was suspected that the increased enzyme output per day in both groups 2 and 4 might be a mere statistical effect of the significantly larger volumes of urine excreted (Table VII) as a result of the greater fluid intake encouraged in these two groups of patients. Since it was very difficult to obtain reliable information about the fluid intake the following, admittedly arbitrary, method was used to test such a hypothesis. The daily enzyme output values of individuals in the normal and " miscellaneous " groups who had excreted a volume of urine of 1700 ml. or more Group ($1 + 3 > 1700$), were statistically compared with the enzyme activity in Groups 2 and 4 (Table VIII). No significant difference was found between the

mean urine volumes in this ($1 + 3 > 1700$) group and the other genito-urinary diseases (2), nor was there any significant difference in the daily enzyme excretion. It was even found that the mean urine volume of group ($1 + 3 > 1700$) was significantly higher than the mean volume of the carcinoma of the bladder group (4), and, correspondingly, the daily enzyme activity in groups ($1 + 3 > 1700$) was higher than in the cancer of the bladder cases.

As an added check, all results from patients with carcinoma of the bladder and other genito-urinary diseases who had excreted a daily volume of less than 1900 ml. (again an arbitrary figure), were combined (group ($2 + 4 < 1900$)) and compared with the enzyme activities in normal subjects (1). There was no significant difference in the enzyme output in these two groups ; the mean volumes of urine excreted were also not significantly different.

The results from Groups 1, 2a, 3a and 4 indicated that, although the mean values were higher in the older groups, age had no significant effect on the urinary β-glucuronidase activity/ml. urine. In both the sub-groups of patients over 50 years of age the mean volumes of urine excreted were less, but not significantly, than in the corresponding main group, and the mean daily enzyme activities were also lower.

In the fracture group, 3b, both the activity per ml. of urine and the daily enzyme output were significantly raised compared with the normal values for at least 10 days after the fracture (Lewis and Plaice, 1959). As the mean volume of urine excreted was lower than normal it was assumed that this was in fact a true difference.

DISCUSSION

The enzyme concentration per ml. of urine is an indication, at any particular time, of the possible hydrolytic opportunities, but the length of time the bladder mucosa is exposed to the carcinogens is another factor which must play a part.

The enzyme activity per ml. of urine was not significantly increased in the cancer of the bladder patients, which is not in accord with the results of Boyland *et al.* (1955). When, however, the enzyme activity per day was considered, both these and the other urinary tract diseases had significantly increased activity compared with the normal value. This increase was not only not specific for the cancer of the bladder patients but it appeared to be a statistical consequence of the greater volumes excreted. This apparent relationship between the volume excreted and the daily enzyme output is difficult to understand.

Boyland and his colleagues (1955) found no β-glucuronidase inhibitors in the urine whereas in the present series 86 per cent of the samples from men and 52 per cent from women had an inhibitor present. The presence of an endogenous β-glucuronidase inhibitor in many types of tissue and in serum is well known (Walker and Levvy, 1953) and an enzyme inhibitor in urine has been demonstrated (Abul-Fadl, 1957). Levvy (1956) points out that an important cause of inhibition of hydrolysis of a particular glucuronide is the presence in urine of other glucuronide conjugates. This will in any case introduce errors of unknown magnitude in the determination of urinary β-glucuronidase activity. It is of interest to note that under the experimental conditions used in this study, the urine of some women contained no inhibitors and appeared to have " activators ". If β-glucuronidase plays an important rôle in releasing bladder carcinogens it is therefore

difficult to understand why women have a lower incidence of bladder cancer than men unless there is some difference between the sexes in the length of time that the carcinogen can act upon the bladder epithelium.

The method used in this work and in the investigation of Boyland *et al.* (1955) entails a dilution of urine with acetate buffer and substrate. This dilution of any inhibitor or " activator " does not give an accurate picture of the enzyme activity in the bladder. Walker and Levvy (1953) discussing an endogenous inhibitor affecting rat-liver β-glucuronidase activity in *in vitro* investigations state that it is by no means certain that the inhibitor has any effect *in vivo*. To study the enzyme in the liver they homogenised the tissue, thus disrupting the cell constituents and perhaps causing contact of enzyme and inhibitor which would not naturally occur. In urine however, it is more likely that the inhibitor is as active in the bladder as it is *in vitro*. The enzyme activity in the bladder is usually less than that estimated *in vitro* at an optimum pH of 4·5. It is possible that the inhibitor or activator action differs at the pH of the urine in the bladder from that under the experimental conditions (Smith and Mills, 1953). Odell and Fishman (1950), investigating the β-glucuronidase activity in human endometrium, found that there was a variation of activity during the menstrual cycle. They found that the lowest values of the enzyme are immediately preceding and following the period of the menses and the highest values occur in the interval between. It seemed possible that these changes in endometrial activity might be reflected in the urinary output although Fishman *et al.* (1951) found no consistent correlation between the blood enzyme activity and the phases of the cycle. The function of β-glucuronidase is still obscure. Not only is it possible that the enzyme might be involved in the synthesis of oestrogen and pregnanediol glucuronides, although this is now considered unlikely, but it might also be involved in structural proliferation of the uterus in the phase before ovulation and in the production of mucin in the secretory phase. In women, cancer of the bladder usually occurs in the post-menopausal stage and the enzyme determinations would not be affected by any cyclical changes. It was thought at the beginning of this investigation that in the control groups, where there was a greater number of younger women, the menstrual cycle might have affected the enzyme activity. If, however, β-glucuronidase plays a rôle in any of the metabolic processes during the menstrual cycle it was not evident in the urinary activity of the small series investigated.

With regard to the increased urinary enzyme activity found after some operations and apparently associated with fairly large amounts of tissue proliferation, Levvy's review (1956) must be borne in mind in which he emphasises that there are many results obtained by different workers which do not support the generalisation that β-glucuronidase activity is invariably related to tissue growth. Although this might be the reason for the elevation during the healing of fractures there may be a relationship between hydrolysis of chondroitin and other mucopolysaccharides and the enzyme activity, as suggested by Levvy (1956).

It is possible that the discrepancy between the results of Boyland *et al.* (1955) and those reported here is due to a difference in the stage of the disease when the patient was investigated. The patients investigated at Southmead Hospital (except for three of the cases in Table IV) were fairly fit and were able to resume normal activities upon discharge after review cystoscopy. In advanced, inoperable cases, even where the pH of the urine is normal, it is feasible that contamination of the urine from the tumour tissue which, Boyland and his colleagues (1955)

have shown to contain high enzyme activity, would cause an increased urinary activity. Our results on the enzyme activity of the urinary sediments from patients with cancer present in the bladder were inconclusive. On the other hand, Boyland's cases may also have had significant bony metastases, another differential feature from ours. It was considered possible that as new bone formation was associated with an increase in the urinary glucuronidase activity, some forms of metastases in bone might have a similar effect. To date a small series of results in patients with such metastases (unpublished) has not been helpful, possibly on account of the steroid hormone therapy given.

Since this was written our attention has been drawn to two recent papers concerned with urinary β-glucuronidase activity in patients with cancer of the bladder. Ohkubo, Sonoda and Kusonoki (1958) in an investigation of the enzyme values of serum, urine and tissue of patients with urological diseases found that in 6 out of 7 cases of bladder cancer the urinary enzyme values were higher than in 16 normal subjects. Mattea and Pietra (1959) observed that in 30 subjects with industrial cancer of the bladder 29 had increased enzyme activity in urine diluted ten times compared with the activity of similarly diluted urine of 4 normal subjects.

The absolute values obtained by the authors of these two papers cannot be directly compared with each other, with the results of Boyland *et al.* (1957) or with the results in the present paper, since experimental factors such as urine and substrate concentration differed.

SUMMARY

1. Urinary β-glucuronidase activity was estimated in patients with cancer of the bladder and in a large number of controls, including patients with other diseases, in addition to normal subjects.

2. β-glucuronidase inhibitors were found in 86 per cent of the men and 52 per cent of the women investigated for this effect.

3. The enzyme activity per ml. urine as well as the daily output were both significantly higher than normal in some cases after major surgery and also, for at least 10 days after fractures.

4. Apart from this the enzyme activity per ml. urine was not significantly different in cancer of the bladder from that in normal subjects or in any of the diseases investigated.

5. The 24-hour output of the enzyme was significantly increased in cancer of the bladder and in other genito-urinary diseases. Data are presented which appear to correlate this increase with the increased volume of urine excreted by such patients.

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REFERENCES

ABUL-FADL, M. A. M.—(1957) *J. clin. Path.*, **10**, 387.

ALLEN, M. J., BOYLAND, E., DUKES, C. E., HORNING, E. S. AND WATSON, J. G.—(1957) *Brit. J. Cancer*, **11**, 212.

BONSER, G. M., BRADSHAW, L., CLAYSON, D. B. AND JULL, J. W.—(1956) *Ibid.*, **10**, 539.

BOOTH, J., BOYLAND, E. AND MANSON, D.—(1955) *Biochem. J.*, **60**, 62.

BOYLAND, E., GASSON, J. E. AND WILLIAMS, D. C.—(1957) *Brit. J. Cancer*, **11**, 120.

Idem, WALLACE, D. M. AND WILLIAMS, D. C.—(1955) *Ibid.*, **9**, 62.

Idem AND WILLIAMS, D. C.—(1956) *Rep. Brit. Emp. Cancer Campgn.*, **34**, 40.

FISHMAN, W. H., KASDON, S. C., BONNER, C. D., FISHMAN, L. W. AND HOMBURGER, F.—(1951) *J. clin. Endocrin.*, **11**, 1425.

Idem, SPRINGER, B. AND BRUNETTI, R.—(1948) *J. biol. Chem.*, **173**, 449.

HUEPER, W. C., WILEY, F. H. AND WOLFE, H. D.—(1938) *J. industr. Hyg.*, **20**, 46.

Idem AND WOLFE, H. D.—(1937) *Amer. J. Path.*, **13**, 656.

LEVVY, G. A.—(1956) ' Vitamins and Hormones ', **14**, 289 (edited Harris, R. S., Marrian, G. F. and Thimann, K. V.). New York (Academic Press Inc.).

LEWIS, F. J. W. AND PLAICE, C. H. J.—(1959) *Nature*, **184**, 1249.

MATTEA, E. AND PIETRA, E.—(1959) *Tumori*, **45**, 86.

ODELL, L. D. AND FISHMAN, W. H.—(1950) *Amer. J. Obstet. Gynec.*, **59**, 200.

OHKUBO, T., SONODA, T. AND KUSUNOKI, T.—(1958) *Urol. int.*, **7**, 167.

PEDERSEN-BJERGAARD, K. AND TØNNESEN, M.—(1948) *Acta endocrin.*, **1**, 38.

SMITH, E. E. B. AND MILLS, G. T.—(1953) *Biochem. J.*, **54**, 164.

TALALAY, P., FISHMAN, W. H. AND HUGGINS, C.—(1946) *J. biol. Chem.*, **166**, 757.

WALKER, P. G. AND LEVVY, G. A.—(1953) *Biochem. J.*, **54**, 56.

WALLACE, D. M.—(1959) ' Neoplastic Diseases at Various Sites ', **2**, ' Tumours of the Bladder '. Edinburgh and London (E. S. Livingstone, Ltd.).

THE ANTIGENIC STRUCTURE OF A TRANSPLANTED SARCOMA

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MURINE iso-antisera have been shown to possess cytotoxic activity when brought into contact with the target cells, *in vitro*, in the presence of active complement (Gorer and O'Gorman, 1956; O'Gorman, 1960). These authors have shown that the cytotoxic iso-antibodies are directed against the H-2 system of antigens, which is known to be the major antigenic system controlling the fate of homografts in mice. The H–2 system comprises a long and complex series of alleles, and our present knowledge of it has been summarised by Gorer and Mikulska (1959).

The iso-antibodies have been shown to be strongly cytotoxic for normal and malignant mouse leucocytes, up to 100 per cent of the target cells being killed (Gorer and O'Gorman, 1956). Other tumours have been found by these authors to be less sensitive to the cytotoxic activity of iso-antisera, for example the C_3H sarcoma BP8 is only partially affected, not more than 60 per cent of the target cells dying after contact. Sarcoma I, on the other hand, has been found to be completely insensitive to the cytotoxic activity of iso-antisera *in vitro*, there being no more non-viable cells in the suspensions treated with antibody than in the normal serum controls (about 5 per cent in each case). The relative susceptibility of three ascites tumours Sa1, BP8 and the leukosis EL4, to one cytotoxic antiserum is shown in Table I. The serum was BALB/c anti-BP8 which contains an antibody against an H–2 component, antigen E, which is common to all three tumours (O'Gorman, 1960).

TABLE I.—*Cytotoxic Effect upon Three Tumours*

Target cells	Percentage non-viable cells at antiserum* dilutions of: Controls		1 : 2	1 : 4	1 : 8	1 : 16
EL4 . .	16	12	95	98	95	91
BP8 . .	1	6	61	49	56	61
Sa1 . .	3	2	5	7	3	9

* Antiserum was BALB/c anti-BP8.

It seemed possible that this apparent complete insensitivity of Sa1 to cytotoxic antibody might be due to some antigenic deficiency on the part of the sarcoma cells. The work now to be reported, in which iso-antisera were absorbed with various tumours and normal liver, and then titrated for residual cytotoxic or haemagglutinating activity, was undertaken to test this theory. It has formed part of a thesis submitted to the University of London for the degree of Doctor of Medicine.

MATERIALS AND METHODS

Serological techniques.—1. The human serum : dextran system described by Gorer and Mikulska (1954) was used for the experiments using haemagglutination.

2. The cytotoxic technique described by Gorer and O'Gorman (1956) and modified by O'Gorman (1960) was used for the cytotoxic experiments. Guinea-pig serum was used to provide complement and trypan blue (1/2000 in Ringer's solution) was the dye.

Mice.—Animals were used from the four inbred lines Strain A, BALB/c, C_3H and C57Bl, and also the so-called E−B+ (H-2g) cross-over, all maintained in the laboratory of Dr. P. A. Gorer.

Tumours.—Two sarcomata were used, Sarcoma I (A strain) and BP8 (C_3H), and one leukosis, EL4 (C57Bl). All three are ascites tumours and will sometimes grow progressively if inoculated intra-peritoneally in large doses, in foreign strains, although they are strain specific when smaller doses are given subcutaneously. Suspensions of these tumour cells were made in Ringer's solution in the case of the leukosis and in 3 per cent sodium citrate for the haemorrhagic sarcomata, by puncturing the distended abdomen with a sterile Pasteur pipette, aspirating the contents and ejecting the fluid into the diluent.

RESULTS

A. *Experiments using cytotoxic technique*

Sera of the type BALB/c anti-C_3H contain the cytotoxic antibodies anti-E, anti-K and anti-D^k. Such a serum, BALB/c anti-BP8, was diluted 1 : 4 and absorbed for 75 minutes at 37° C. with equal volumes of BALB/c liver, and packed cells of EL4, BP8 and Sa1 ascites. The absorbed sera were tested on EL4. The only antibody which was being tested was therefore anti-E. The titre of the unabsorbed serum was 1 : 64, and that of the serum absorbed with BALB/c liver was also 1 : 64. No residual cytotoxicity was found after absorption with EL4 and BP8, but absorption with Sa1 left cytotoxic activity up to a titre of 1 : 32. Both EL4 and BP8 had therefore absorbed anti-E, as would be expected. Sa1, on the other hand, had not absorbed anti-E and the tumour cells were presumed to lack E.

This experiment gave no information as to the amount of anti-E which would be absorbed by normal A cells, and therefore a further experiment was performed in which the same BALB/c anti-C_3H serum was absorbed with C liver, A liver and Sa1 and tested on EL4. The antibody is again anti-E. On this occasion the titre of both the unabsorbed serum and that absorbed with C liver were 1 : 128. No cytotoxicity was left after absorption with A liver, but absorption with Sa1 left a cytotoxic titre of 1 : 32. It was concluded that Sa1 contains less E antigen than does A liver. However, absorption with Sa1 lowered the titre by 2 tubes compared with BALB/c liver. This might indicate that Sa1 is not totally lacking in E; on the other hand, this absorption may be due to the presence of normal A strain cells (probably mainly red blood cells) in the Sa1 ascites.

B. *Experiments using haemagglutination technique*

A sample of BALB/c anti-EL4 (containing anti-D^b and anti-E) was absorbed at various dilutions with one equal volume of A liver. At a dilution of 1 : 32 all

the anti-E was absorbed and no haemagglutination was detected for A strain or C_3H red blood cells. At this dilution absorption with Sa1 left a titre of 1 : 2048 for C_3H cells and with BALB/c liver left a titre of 1 : 4096, both due to unabsorbed anti-E.

A serum was produced in the E−B+ (H–2g) crossover against EL4, containing anti-E and anti-K^b. When tested against A and C_3H red cells, only anti-E is involved. The unabsorbed serum gave a titre of 1 : 16,384 for A cells and 1 : 4096 for C_3H; absorption with BALB/c liver lowered the titre to 1 : 4096 for A and 1 : 2048 for C_3H. Absorption with Sa1 left antibody with the same titre as BALB/c liver, but A liver almost cleared the antibody, leaving only a titre of 1 : 64 for A cells and less than 1 : 16 for C_3H.

In an attempt to get some estimate of the amount of E present on the Sa1 cells the absorptive power of Sa1 for anti-E was compared with that of decreasing volumes of A liver. To do this satisfactorily a minimum amount of antibody must be used, so that one is working at the end of the titre. When E−B+ anti-EL4 was diluted 1 : 256 and absorbed with 0·1 volume of A liver the anti-E remaining only agglutinated A strain erythrocytes in the first tube, i.e. at a dilution of 1 : 512. 1·0 volume of Sa1, however, absorbed more anti-E than 0·1 volume of A liver, for there was no residual agglutination of A cells at 1 : 512.

This same serum was then diluted 1 : 64 and absorbed with 0·1 or 0·2 volume of A liver and with 1·0 volume of Sa1. In this case the titre for A cells was reduced from 1 : 4096 for the unabsorbed serum to 1 : 128 by absorption with 0·2 volume of A liver and to 1 : 256 with 0·1 volume. 1·0 volume of Sa1 lowered the titre to 1 : 128. It was concluded that Sa1 packed ascites cells contain only about 20 per cent of the E antigen which is contained in normal A strain liver cells.

Similar experiments were done to test for the presence of the K and D antigens on Sa1 cells. Sera produced in the (BALB/c × C57Bl)F_1 against A strain tumour contain only anti-K. Such a serum was absorbed with one volume of A liver or Sa1 at various dilutions. When diluted 1 : 4, 1 : 8 or 1 : 16 absorption with A liver reduced the titre from 1 : 1024 for unabsorbed serum to 1 : 16 for the first dilution and less than this for the other two. Sa1 reduced the titre to 1 : 512, 1 : 256 and 1 : 128 for the dilutions 1 : 4, 1 : 8 and 1 : 16 respectively. This shows that Sa1 contains much less K antigen than does A liver.

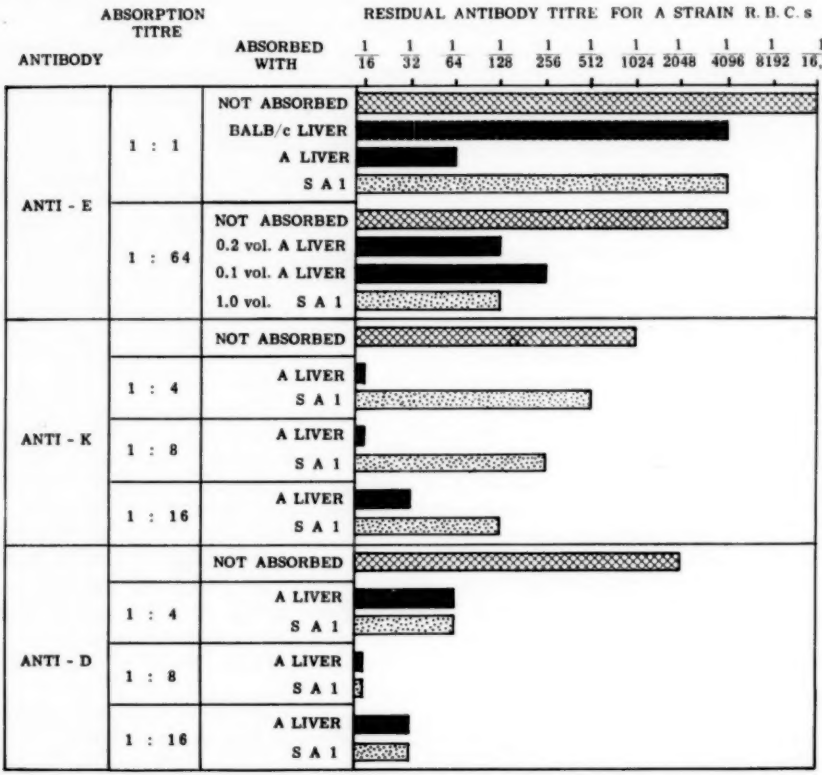
The results of testing for antigen D showed no significant difference between the amount on Sa1 and A liver cells. The serum used was made in the (C_3H × C57Bl)F_1 against A strain tumour, and contained only anti-D. The antibody was absorbed and tested in the same way as anti κ. Both Sa1 and A liver left similar titres at all three dilutions. They were 1 : 64, 1 : 16 and 1 : 32 after absorption at 1 : 4, 1 : 8 and 1 : 16 respectively.

The results of these absorptions are expressed graphically in Fig. 1. They show that Sa1 packed ascites cells contain about 20 per cent of the E antigen and much less K than normal A strain liver cells, but approximately the same amount of D.

DISCUSSION

Snell (1957) has reviewed the work on the different types of reaction to tumour grafts, and has propounded a classification of transplantable tumours based on their susceptibility to the action of antibody, dividing them into three classes. The first includes tumours which are completely susceptible to the action of anti-

body, such as EL4. The second comprises tumours which are partially susceptible, such as $6C_3HED$, which tend to respond to the action of antibody by enhanced rather than inhibited growth. Sa1 falls into this group. The third group are those which are quite insensitive to the action of antibody, for example the adenocarcinoma D1905.



EQUAL VOLUMES OF LIVER OR S A 1 PACKED CELLS WERE USED FOR ABSORPTION UNLESS OTHERWISE STATED

FIG. 1.—Absorption of iso-antibodies by Sa1.

The results of the extensive absorption experiments reported above show that Sarcoma I growing in the ascites form in the Strain A mice maintained in Dr. Gorer's laboratory possesses about 20 per cent of the antigen E possessed by a corresponding amount of normal A liver; it also possesses less of the K antigen than does A strain liver, but about the same amount of antigen D. There appear to be three possible explanations for this antigenic loss. The first is that all the Sa1 cells are deficient of the antigens by the same amount, whilst the second is that some 80 per cent of the cells are wholly deficient of antigen E and the remaining 20 per cent carry the normal amount; similarly K might be partially missing

from all the cells, or totally missing from some. The third possibility is that the sarcoma cells are all deficient of these antigens and their apparent presence is due to contamination of the ascites with normal erythrocytes and leucocytes having normal antigenic constitutions. We have no definite evidence for or against any one of these theories but as Sa1 ascites always contains a proportion of host erythrocytes and leucocytes it seems reasonable to deduce that Sa1 cells are wholly deficient of antigens E and K, and that the small amounts found are due to the normal cells in the ascites. We can only speculate upon the possible relationship between this antigenic loss and the readiness with which the growth of Sa1 may be enhanced by the action of antibody on the one hand, and on other, the insensitivity of Sa1 cells to the *in vitro* toxic effect.

Kaliss (1958) believed that immunological enhancement is due to a direct effect of antibody upon the tumour cells. This is evidently not a toxic but rather a stimulating effect, so that the tumour is altered in some way and is thus better able to withstand and overcome the host's defensive reaction. Sarcoma I may be insensitive to cytotoxic antibody because of its antigenic deficiencies, but this need not preclude a stimulant effect of the antibody on the cell and stimulation of an antigen deficient cell by antibody may lie at the root of the mechanism of immunological enhancement.

SUMMARY

Experiments have been reported which have shown, by means of graduated absorption of iso-antisera, that suspensions of Sa1 show marked deficiencies of the H–2 antigens E and K, but no deficiency of antigen D. It is possible that these deficiencies are connected with the lack of sensitivity of Sa1 to cytotoxic antibody and the ease with which the growth of the tumour may be enhanced.

The authors wish to express their gratitude to Dr. P. A. Gorer for his help and advice during this investigation.

REFERENCES

GORER, P. A. AND MIKULSKA, Z. B.—(1954) *Cancer Res.*, **14**, 651.—(1959) *Proc. Roy. Soc.* B, **151**, 57.

Idem AND O'GORMAN, P.—(1956) *Transpl. Bull.*, **3**, 192.

KALISS, N.—(1958) *Cancer Res.*, **18**, 992.

O'GORMAN, P.—(1960) *Brit. J. Cancer*, **14** (in press).

SNELL, G. D.—(1957) *Cancer Research*, **17**, 2.

EFFECT OF LETHALLY DAMAGED TUMOUR CELLS UPON THE GROWTH OF ADMIXED VIABLE CELLS IN DIFFUSION CHAMBERS

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IT has been shown that the proliferation of a small viable tumour graft is stimulated by the presence of irreversibly X-ray damaged tumour cells (Révész, 1958) or viable but genetically incompatible cells (Klein and Klein, 1956). Histological examination (Ringertz, Klein and Révész, 1959) showed an enhanced granulation tissue formation in and around the implant. The intensity of this reaction was parallel to the stimulating effect of X-ray damaged, genetically incompatible, and heat-killed cells, respectively. This would indicate that the stimulating function may depend on the formation of a proper tumour bed. In addition, a direct " feeder " effect (Puck, Marcus and Cieciura, 1956) may also play a certain role since heavily irradiated cells were stimulatory even in the case of freely suspended ascites tumour cells (Révész, 1955 ; Scott, 1957 ; Mazurek and Duplan, 1959).

The diffusion chamber technique of Algire, Weaver and Prehn (1954) permits the isolation of the graft from direct contact with host cells. Filter membranes with adequately small pores permit the diffusion of soluble materials but prevent the outbound passage of graft cells and inbound movement of the host cells. Grafts of various kinds have been shown to survive and proliferate for long periods of time under such conditions (Algire *et al.*, 1954 ; Shelton and Rice, 1958 ; Amos and Wakefield, 1958 ; and others).

In the present paper the growth of untreated ascites tumour cells was measured alone or in the presence of lethally damaged cells within diffusion chambers in an attempt to clarify the role of the cellular host reaction in the stimulation phenomenon.

MATERIALS AND METHODS

Mice : Various F_1 hybrids were used, obtained by mating males of the A.SW strain (Snell, 1955) with females of the strains C3H/Kl, DBA/2/Kl and C57BL/Kl. The animals weighed 20–26 g. and were 2–4 months old. They were maintained on a standard pellet diet which, together with drinking water, was available *ad libitum*.

Tumours : The Ehrlich/Sto ascites tumour (Klein and Révész, 1953), propagated in hybrid mice was used in all experiments except one with the L1210 ascites lymphoma of the DBA/2 strain (Law *et al.*, 1949). In some experiments, the following two tumours were admixed with cell suspensions of the Ehrlich carcinoma : Ascites sarcoma MC1M of strain C3H origin (Klein, 1951) and the solid fibrosarcoma MSC induced by methylcholanthrene in an A/Sn mouse.

The ascites tumours were propagated by weekly intraperitoneal transfers of 0·1 ml. ascites fluid diluted 1 : 10 in Ringer's solution. The solid neoplasms were

suspended in Ringer's solution after having been forced through a stainless steel screen and were propagated by subcutaneous transfer.

Irradiation: X-rays were generated in a Siemens X-ray machine at 185 kv and 15 mA, and were filtered by 1 mm. Al. Irradiation of the tumour suspensions was performed *in vitro* in a sterile flat-bottomed plastic irradiation chamber with an inside diameter of 26 mm. and a height of 24 mm. The suspensions, each approximately 5 ml., were irradiated with 12000 r at a rate of 425 r/min; the distance from the focus of the X-ray tube to the bottom of the vial was 29·5 cm.

Chambers: The diffusion chambers used in the present investigation were designed after the chambers of Algire *et al.* (1954). They consisted of two parts: (1) a "cup", constructed of a disc of HA millipore filter (Millipore Filter Corp., Watertown, Mass.) sealed to the bottom of an acrylic cylinder (material manufactured by R. Daleman, Ltd., London) having an outside diameter of 16·8 mm., an inside diameter of 14·5 mm. and a height of 5·0 mm.; (2) a "lid", with a construction similar to that of the cup, but with larger dimensions, namely 19·5 mm. outside diameter, 17·0 mm. inside diameter and 5·0 mm. height. When adjoined they composed a closed chamber having a volume of 0·8 ml. In one experiment (with L1210 lymphoma) chambers of half of this volume were used; they had the same diameter but a height of only 2·5 mm.

The HA millipore filter was employed in all experiments with an average pore size of 0·45 μ. The filter was sealed to the acrylic cylinders with acetone in which filter material had been dissolved (about 4 filters to 10 ml. acetone). The cups and lids were sterilized by immersion into 70 per cent alcohol for 6 hours. Subsequently they were placed in sterile Petri dishes and dried in a desiccator.

Transfer procedure

Transfer of the tumour cell suspensions into the chambers was performed under aseptic conditions in a room assigned for sterile work. The cups, usually 10 of them at a time, were placed in a row on a sterile metal plate and 0·2 ml. ice-cooled tumour suspension, appropriately diluted in Ringer's solution, was pipetted into each cup. The lids were then drawn over the cups with a forceps, the chambers were inverted and their two parts were sealed by a commercial fast drying lime (Karlssons Klister, AB Klärre, Stockholm) at their junction. Usually thirty to forty chambers were prepared in an experimental series by a single operator. The closed chambers were kept in ice-cooled Ringer's solution until insertion into the peritoneal cavity of the mice.

The animals were anaesthetized with 0·006 ml. per g. bodyweight of a 1 per cent Nembutal solution injected intraperitoneally. The abdominal skin was washed with a cotton wool pledget moistened with alcohol, and an incision of about 2 cm. length was made. The chamber was inserted into the peritoneal cavity and the abdomen was closed by a continuous silk peritoneal suture and agraff skin clips. Depending upon the nature of the experiment between 2 and 6 hours were needed to complete the procedure. During this period the suspensions were kept in an ice bath at 4° C.

Sampling procedure

Each animal was killed by cervical dislocation, the abdomen was opened and the chamber was carefully removed from the peritoneal cavity. A coat of

accumulated host cells was rubbed off the external surfaces by a filter paper. Subsequently, the chamber was either used for the quantitative determination of its cellular content, or for sterility and viability tests, respectively. Different chambers inoculated with the same pool of cells were assigned for these two procedures in a random way.

Quantitative sampling: Each chamber assigned for quantitative determination of its total cell content was covered with a commercial nailpolish over its entire surface in order to trap the host cells adhering to the outer surface and to prevent them from intermixing with the chamber contents. The chamber was fixed in a small holder apparatus and opened on one side by cutting it with a pointed forceps around the edges of the millipore filter. After the incubation periods used in this study, all chambers were found to be completely filled with a viscous fluid in which clots were frequently observed.

Following opening the entire chamber was submerged into a flat-bottomed glass vial containing 4 ml. of aqueous solution of 0·1 M citrate and 0·5 per cent crystal violet. The vial was plugged with a rubber stopper and shaken at a frequency of about 1200 oscillations per minute and an amplitude of 0·8 cm. for 5 minutes in a microid flask shaker (Griffin and Co. Ltd., London). This treatment dissolved occasional clots and provided a homogeneous suspension of stained cells. Microscopical examination of the filters indicated that the shaking treatment was also effective in removing all cells from the inside of the wall of the chambers.

The cell concentration of the stained suspension was determined in undiluted or diluted (1 : 10) aliquots in a Buerker haemocytometer. At least 50 cells were counted in 4 $\times$ 1 mm^3 undiluted fluid. In diffusion chambers with a volume of 0·8 ml., this cell number corresponds to a total population of 6 $\times$ 10^4 cells. The population size of chambers containing less than this number was not determined.

The proportion of cells containing nuclei stainable with crystal violet was estimated by differential counts on the material in the haemocytometer. At least 200 cells were counted. Absent or indistinct nuclear staining with dispersed and/or fragmented chromatin debris was taken as evidence for irreparable cellular damage. The proportion of such cells was taken as the minimum limit of non-viable cells in the population.

Sterility and viability tests: The chamber was removed from the peritoneal cavity and cleaned from the coating capsule. Fixed in the holder apparatus, one of the chamber membranes was perforated by the point of a Pasteur-pipette. A small drop of the chamber contents was transferred into a bouillon broth and incubated for three days at 37° C. If bacterial growth was obtained, all chambers inoculated from the same pool were rejected. Usually an experimental series consisted of six chambers out of which two were used to test bacterial contamination.

In addition to the sterility test, the same chamber was also used to estimate the approximate proportion of viable cells by the procedure of Schrek (1936). In order to bring all tumour cells into a homogeneous suspension, the open chamber was transferred into a glass vial, containing 4 ml. ice-cooled Locke's solution which was shaken for 5 minutes. The possible damage caused by this treatment to viable cells was not determined. The eosin-unstained fraction of cells was therefore considered as a minimum estimate of the originally viable fraction.

A comparison of the results obtained by the crystal violet procedure, using nuclear staining as criterion of viability, and the Schrek test, shows a positive

correlation although the Schrek test gave usually a lower estimation of the size of the viable fraction (Fig. 1).

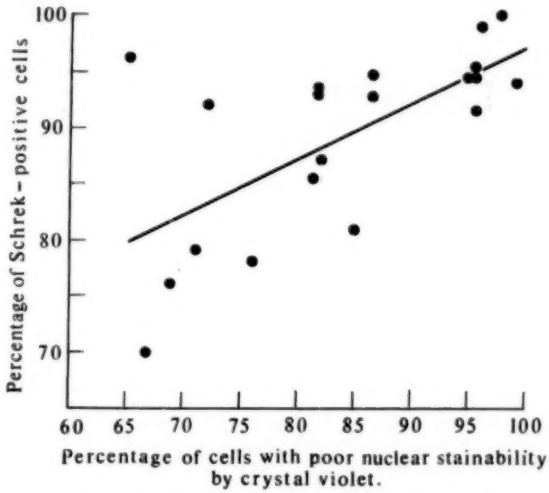

FIG. 1.—The percentage of Schrek-positive cells as correlated with the proportion of cells with poor nuclear stainability in the crystal-violet test. Each point represents a single chamber, at different times after the introduction of varying numbers of viable Ehrlich ascites tumour cells ($n = 20$, $r = +0{\cdot}635$, $0{\cdot}001 < P < 0{\cdot}01$).

RESULTS

I. *Proliferation of Ehrlich ascites tumour cells in diffusion chambers*

This was investigated by quantitative determination of the population size at different times after the introduction of various cell numbers (Table I).

Aliquots of 0·2 ml. Ringer solution containing 10^2 to 10^6 viable Ehrlich ascites tumour cells were pipetted into each chamber. The experiments with the smallest inoculum (10^2 in series 1, Table I) was repeated 6 times, while the other series (series 2–5) are based on one experiment each. The total number of cells was determined on the 20th and 30th day after implantation, with the exception of series 1 and series 5 (cf. column 4, Table I). Only chambers containing more than 6×10^4 cells were evaluated. About 50 per cent of the samples implanted with 10^2 cells were omitted since they contained fewer cells. The standard deviation of the mean cell number is shown in column 7 in Table I. It is inversely related to the inoculum size. A similar relationship was found for ascites tumours growing freely in the abdominal cavity (Klein and Révész, 1953).

The results are illustrated in Fig. 2. It can be seen that the tumour cells have grown to multiples of the initial population size during the cultivation period. The ratio of the mean cell number and the inoculum dose was inversely related to the latter (column 8, Table I). This may be due to a gradual decrease of the relative multiplication rate with increasing size as found with freely growing ascites cells (Klein and Révész, 1953). The largest population size, approximately

TABLE I.—*Data from Diffusion Chamber Experiments with Viable Ehrlich Ascites Tumour Cells*

Series number	Number of separate experiment	Inoculum number of tumour cells	Time after inoculation (days)	Number of chambers used for separate determinations	Mean population size (log. units)	Standard deviation (log. units)	Ratio of mean population size and inoculum (log. units)
1	2	3	4	5	6	7	8
1	6	10^2	15	12[1]	5·28	0·650	3·28
			30	15[2]	5·72	0·908	3·72
2	1	10^3	20	5	5·65	0·594	2·65
			30	5	6·38	0·245	3·38
3	1	10^4	20	5	6·36	0·301	2·36
			30	5	6·46	0·406	2·46
4	1	10^5	20	5	6·52	0·257	1·52
			30	5	6·77	0·239	1·77
5	1	$1·8 \times 10^6$	2, 6, 9, 12, 30	20	6·91[3]	0·064[5]	0·91[3]
					6·98[4]	—	0·98[4]

[1] Eleven chambers with less than 6×10^4 cells are not included.
[2] Ten chambers with less than 6×10^4 cells are not included.
[3] On the 12th day.
[4] On the 30th day.
[5] Established from duplicate determinations at the intervals indicated in column 4.

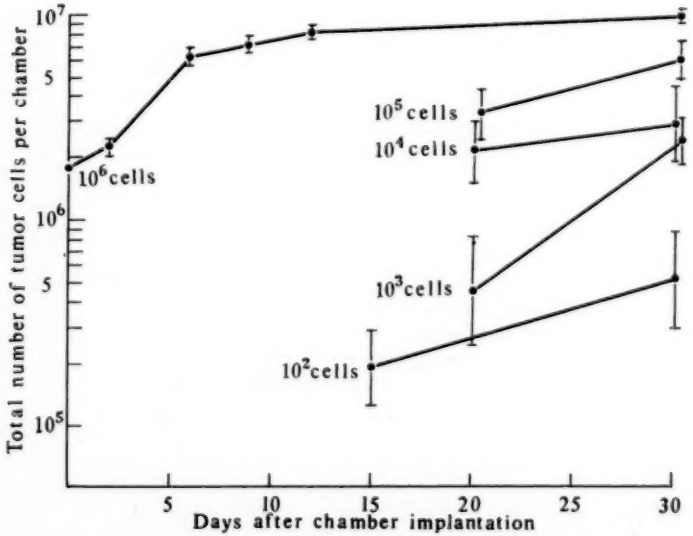

FIG. 2.—Growth of different doses of Ehrlich ascites tumour cells in diffusion chambers. The mean and its standard error is indicated. Each point represents the mean of 5–15 chambers.

$9{\cdot}5 \times 10^6$ cells per chamber, was attained after the inoculation of $1{\cdot}8 \times 10^6$ cells under the conditions used. This corresponds to a concentration of about $1{\cdot}3 \times 10^6$ cells per 0·1 ml. chamber fluid.

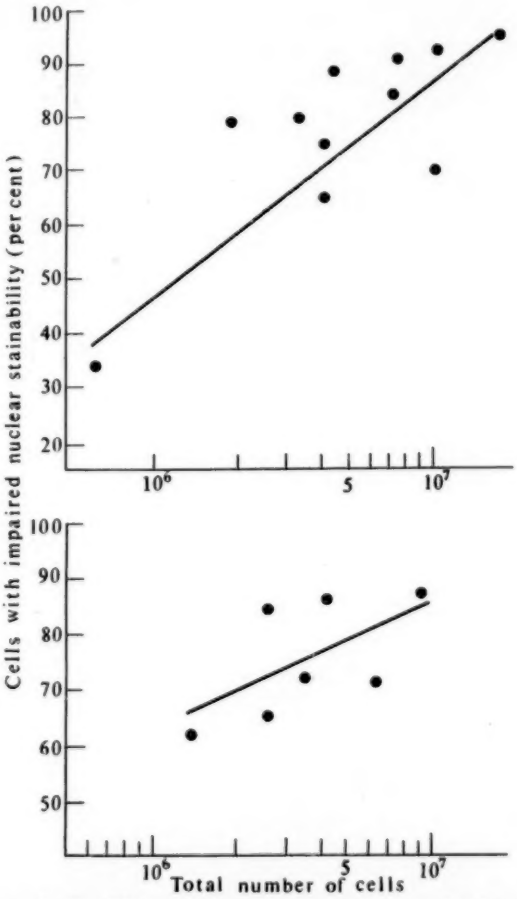

FIG. 3.—Percentage of cells with impaired nuclear stainability as related to population size in the chamber. Differential counts were made on crystalviolet stained material. The upper diagram represents chambers harvested 20 days after the introduction of 10^3 Ehrlich ascites tumour cells ($n = 7$, $r = 0{\cdot}656$, $0{\cdot}05 < P < 0{\cdot}1$), while the lower diagram corresponds to 30 days and 10^4 or 10^5 inoculated cells ($n = 12$, $r = 0{\cdot}684$, $0{\cdot}01 < P < 0{\cdot}02$).

The results of differential countings on crystal violet stained material indicate that the proportion of cells with far advanced nuclear damage increased with time (Fig. 3). While the great majority of the free Ehrlich ascites cells appear to be viable during the entire growth period (Klein and Révész, 1953), the percentage of damaged cells increases in the chambers with time.

II. *Behaviour of heavily irradiated and heat-killed cells in diffusion chambers*

Two separate experiments were performed in order to study the viability of heavily irradiated (HR) cells at varying intervals after introduction of 10^7 HR cells suspended in 0·2 ml. Ringer solution. The eosin test of Schrek indicated that the unstained fraction decreased progressively until after seven days the overwhelming majority of the population became eosin stainable (Fig. 4). Occasionally eosin-unstained cells of considerably increased size were discernible as long as 16 days after incubation.

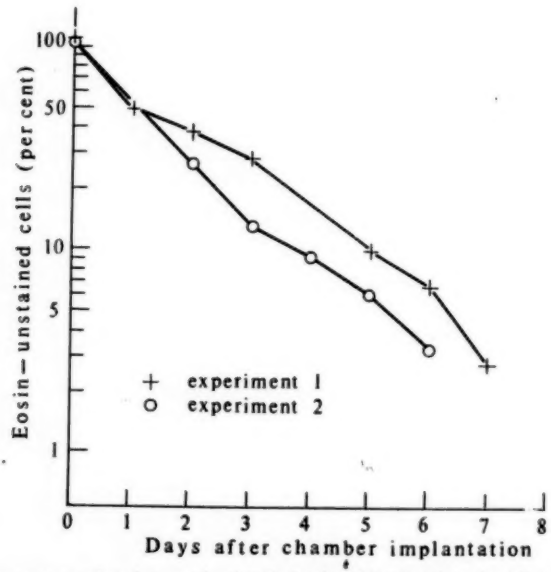

FIG. 4.—The percentage of eosin-unstained cells in chambers incubated with 10^7 HR Ehrlich ascites cells. Each point represents a single determination in one chamber (two separate experiments).

Disintegration of the HR cells proceeded at a slow rate. In 3 separate experiments (series 6, Table II) using an HR inoculum of 5×10^5 Ehrlich cells per 0·2 ml. about 90 per cent of the original population could be recovered on the 15th day, and almost 40 per cent on the 30th day (column 9, Table II). When suspended in citrate solution and stained with crystal violet, the recovered cells showed an indistinctly staining or completely dissolved nucleus. If chambers were incubated with 10^7 HR cells for 150 days (series 7, Table II), about 30 per cent of the original inoculum were found in the fluid as diffusely stained cellular entities.

In two separate experiments (series 8, Table II) the disintegration of heat-treated cells was followed. As the HR cells, they disintegrated at a slow rate. Approximately 70 and 50 per cent of an initial number of 5×10^5 heat-killed cells could still be identified after 15 and 30 days, respectively.

TABLE II.—*Data from Diffusion Chamber Experiments with Viable and/or Treated Tumour Cells*

		Inoculum								
			Treated cells							
Series number	Number of separate experiments	Untreated Ehrlich ascites cells	Type	Treatment	Number	Time after inoculation (days)	Number of chambers used for separate determinations	Mean population size (log. units)	Standard deviation (log. units)	Ratio of mean population size[1] and untreated inoculum (log. units)
1	2	3	4	5	6	7	8	9	10	11
1	6	10^2	Ehrlich	12,000 r	5×10^5	15	25	6·53	0·466	4·53
						30	14	6·75	0·397	4·75
2	4	10^2	Ehrlich	Boiling water bath 10 min.	5×10^5	15	15	5·79	0·509	3·79
						30	4	6·19	0·182	4·19
3	1	10^2	MC1M	12,000 r	5×10^5	15	4	6·30	0·314	4·30
						30	2	6·21	—	4·21
4	1	10^2	MSC	12,000 r	5×10^5	15	3	6·39	—	4·39
						30	3	6·54	—	4·54
5	1	10^2	Liver	12,000 r	5×10^5	15	3	6·43	—	4·43
6	3	—	Ehrlich	12.000 r	5×10^5	15	9	5·66	0·146	—
						30	11	5·28	0·263	—
7	2	—	Ehrlich	12,000 r	10^7	150	5	6·46	0·211	—
8	2	—	Ehrlich	Boiling water bath 10 min.	5×10^5	15	6	5·56	0·194	—
						30	4	5·42	0·146	—

[1] Population size after substraction of initially admixed treated cells.

III. *Admixture of heavily irradiated tumour cells to a small viable inoculum*

A large number (5×10^5) of HR Ehrlich ascites tumour cells in 0·2 ml. Ringer solution was admixed with 10^2 viable cells in another 0·2 ml., and introduced into the chamber (series 1, Table II). On the 15th and 30th day the total cell number was determined in 25 and 14 chambers, respectively. Correction was made for the initial number of treated cells by substracting it from the total number. This was considered necessary since both irradiated and heat-killed cells could maintain themselves in the chamber nearly quantitatively during the observation period. Since some of them may have disintegrated, the correction employed may have introduced a slight underestimation of the extent of growth.

A comparison between series 1 in Table I and series 1 in Table II indicates that 10–20 times larger cell populations were attained in the presence of HR cells than in their absence. This must be regarded as a minimum estimate since about half of the control chambers inoculated with small viable inocula alone contained less than 6×10^4 cells and therefore were eliminated from the calculations. Even so the differences were highly significant (day 15 : $P < 0{\cdot}001$; day 30 : $0{\cdot}01 > P > 0{\cdot}001$).

Similar results were obtained when the lymphoma L1210 was grown in 0·4 ml. diffusion chambers, either in the presence or in the absence of HR cells of the same kind. The inocula contained 10^2, 10^4 or 10^6 viable cells suspended either

in 0·1 ml. Ringer solution alone or in 0·2 ml. Ringer containing 10^5 HR cells. The chambers were harvested and their total cell content was determined after 8, 16 and 25 days. Correction was made by substracting the number of added HR cells from the figures. Fig. 5 shows that considerably larger populations were obtained in the presence of HR cells in this case too. The multiplication of the lymphoma cells decreased with increasing population size and approached a maximal value of about 150×10^6 total cells per chamber. This corresponds to a concentration of about 36×10^6 cells per 0·1 ml.

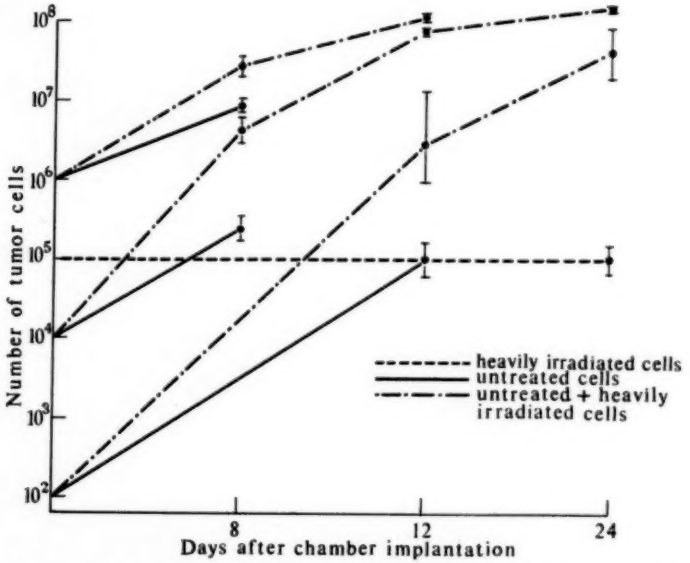

FIG. 5.—Cell content of diffusion chambers after inoculation of 10^2, 10^4 and 10^6 L1210 lymphoma cells either alone or together with 10^5 HR cells of the same kind. The points represent means of 2–6 determinations, and the range is indicated. The number of irradiated cells was subtracted from the actual figures.

IV. *Admixture of heat-killed tumour cells to a small viable inoculum*

Ehrlich ascites tumour cells were suspended in Ringer's solution to a concentration of 5×10^5 per 0·2 ml. and killed by immersion into boiling water for 10 minutes. This suspension was mixed in 0·2 ml. aliquots with another 0·2 ml. Ringer solution containing 10^2 viable Ehrlich cells and introduced into diffusion chambers in the usual way (series 2, Table II). The cell contents of the chambers were determined after 15 and 30 days. The initial number of heat-killed cells was substracted from the total number.

Somewhat larger cell populations were attained in the presence of heat-killed cells than in their absence (series 1 in Table I and series 2, Table II). The differences were not significant: $P > 0{\cdot}1$ after 15 days, $P \sim 0{\cdot}3$ after 30 days. The difference between the HR group and the heat-killed cell admixture was

significant after 15 days ($P < 0.001$) but not after 30 days ($P \sim 0.3$) (series 1 and 2 in Table II).

V. *Effect of foreign tumour or liver cells*

To study the possible specificity of the stimulating effect, 10^2 viable Ehrlich cells were introduced into the chamber together with 5×10^5 HR cells derived either from the MC1M ascites sarcoma or from the solid MSC fibrosarcoma (series 3 and 4, Table II). The size of the population was determined after 15 and 30 days, after subtracting the initial number of foreign irradiated cells. After 15 days the corrected cell numbers were about 10 times larger than in the controls (series 1, Table I) ($0.01 > P > 0.001$). After 30 days the mean cell numbers were still larger than the controls but the difference was not significant ($P > 0.3$).

In one experiment (series 5, Table II) 10^2 Ehrlich cells were grown in the presence of approximately 5×10^5 cell of an A.SW mouse liver suspension. On the 15th day the size of the tumour cell populations was determined in 3 chambers after differential counting tumour and liver cells. A stimulating effect was observed, comparable to that observed with heavily irradiated Ehrlich cells.

DISCUSSION

The number of Ehrlich ascites tumour cells increases at a slower rate inside the diffusion chambers than in the peritoneal fluid. A comparison of the growth curve of an inoculum of 1.8×10^6 Ehrlich ascites tumour cells in the peritoneal cavity (Klein and Révész, 1953) and in the chambers shows a nearly logarithmic correlation (Fig. 6). Whereas the percentage of eosin-stainable cells was negligible throughout the entire period of ascites growth in the peritoneal cavity (Klein and Révész, 1953) an increasing number of dead and degenerating cells could be found in the chambers, however.

The differences between the intraperitoneal and intrachamber growth can be assumed to be due to the fact that the diffusion barrier leads to a slower flow of nutrients and an accumulation of waste products. The entry of fluid into the chambers has been studied by Amos and Wakefield (1958). They placed empty chambers, corresponding in physical characteristics to those used in the present investigation, into the peritoneal cavity of mice in order to study the rate of fluid passage. After a lag of about 6 hours, filling proceeded at a rate of 0.085 ml. per day and after 7 days the chambers were filled. When high titer isoantiserum was injected intraperitoneally an equilibrium was obtained, usually after about 90 minutes, between the haemagglutinin levels of the peritoneal fluid and the chamber contents.

It has been found that the growth of the Ehrlich ascites carcinoma in the peritoneal fluid is characterized by a progressively decreasing multiplication rate, coming to a stand-still after a maximum population had been reached. Qualitatively similar growth curves were obtained in the chamber. The maximum cell concentration of Ehrlich ascites tumour cells was about 1.3×10^6 cells per 0.1 ml. in the chamber; this is approximately one tenth of the cell concentration in the free ascites fluid (Klein and Révész, 1953). With the L1210 lymphoma, however, the concentration in the chamber, 36×10^6 cells was similar to the values found with this tumour in the ascites fluid (Shelton and Rice, 1958*b*). A third ascites tumour, ELD, reached one third of its usual cell concentration in the chamber

(11×10^6 cells per 0·1 ml.) (Norman, not yet published). These differences appear to indicate that different tumours may vary considerably in their resistance to adverse conditions.

Disintegration of dead cells was found to proceed at a very slow rate in the chamber. When lethally irradiated or heat killed cells were introduced, a considerable proportion of the original inoculum was distinctly discernible for more than four weeks. In her experiments with different lymphomas grown in diffusion chambers, Shelton found that soon after incubation dead cells became

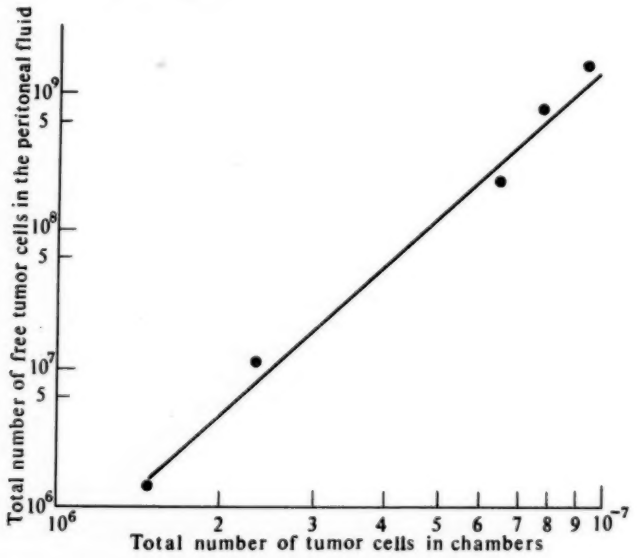

FIG. 6.—Correlation between total cell number at corresponding times in the peritoneal fluid and in the chamber after inoculating $1·8 \times 10^6$ Ehrlich ascites tumour cells intraperitoneally or into the chamber, respectively. Regression line coefficient: 3·59. Chambers of 0·8 ml. volume were used.

a large component of the population (Shelton and Rice, 1958*a*). It would seem that the chamber fluid has little, if any, proteolytic activity, perhaps due to the absence of inflammatory cells.

In contrast to this situation, radiation damaged cells in direct contact with host cells disintegrate soon after implantation. Inflammatory but no tumour cells were found in the ascites fluid of mice 6 days after intraperitoneal injection of Ehrlich ascites cells irradiated with 4000 r (Révész, 1955). Genetically compatible sarcoma cells treated with 12000 r and injected subcutaneously, were found embedded in a granulation tissue until the 7th day, but not later (Ringertz *et al.*, 1959). It would seem that disintegration of irradiated tumour cells at either a subcutaneous or intraperitoneal implantation site occurs at about the same time that the majority of similarly treated cells introduced into chambers become eosin-stainable (Fig. 4).

The stimulating effect of irradiated cells upon the growth of admixed viable cells as observed in the chambers is in conformity with analogous findings with ascites cells in the peritoneal fluid (Révész, 1955; Scott, 1957; Mazurek and Duplan, 1959) and with various tumours in the subcutaneous tissue (Révész, 1958). Two possible mechanisms have been considered previously to explain the phenomenon: a "feeder" effect (Puck *et al.*, 1956) based upon the release of nutrients and/or growth stimulating substances or, alternatively, an effect mediated through *local* host responses. The possibility of a *systemic* host effect was excluded since no stimulation was obtained when viable and heavily irradiated (HR) cells were inoculated at two different anatomical sites (Révész, 1958). Histological studies of the implantation site actually revealed a certain correlation between the ability of a given cell preparation to provoke an intense granulation reaction and its stimulating effect (Ringertz *et al.*, 1959). It was concluded that the host reaction may be an important factor acting probably by enhancing vascularization and stroma formation.

The chamber experiments show that HR cells can stimulate even in the absence of any cellular host reaction. This is analogous with the finding that single HeLa cells *in vitro* show an increased plating efficiency and accelerated growth rate if plated in the presence of a "feeder layer" of X-irradiated cells (Puck *et al.*, 1956).

The stimulating effect does not appear to be tumour specific since heavily irradiated suspensions of foreign mouse tumours and a suspension of liver tissue are also effective. This finding is in harmony with the previous observations that growth of small subcutaneous inocula of different mammary carcinomas and methylcholanthrene-induced sarcomas can be stimulated by admixed suspensions prepared from liver (Révész, 1958), or embryonic tissue (Schneyer, 1955; Vasiliev and Olshevskaja, 1958) or from different tumours (Klein and Klein, 1956). It is at variance with the findings of Mazurek and Duplan (1959) who maintain that stimulation is specific for the cells of the same tumour.

At the subcutaneous site heat-killed tumour cells exhibited no stimulating effect (Révész, 1958). In the chambers heat-treated cells showed a certain stimulating activity which was, however, significantly less than that of radiation-killed material.

SUMMARY

The influence of a large number of irreversibly damaged tumour cells upon the subsequent proliferation of a small, admixed viable cell fraction was studied in diffusion chambers inserted into the peritoneal cavity of mice. A method is described for quantitative estimation of the size of the cellular population in the chambers. A considerable proportion of the original population of X-ray inactivated or heat-killed cells was distinctly discernible in the chambers for more than four weeks. Viable cells admixed to heavily irradiated material of the same or different tumours or to liver tissue were found to grow more rapidly than the same number of viable cells cultured alone. Heat-killed cells enhanced the growth to a significantly less extent.

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REFERENCES

ALGIRE, G. H., WEAVER, J. M. AND PREHN, R. T.—(1954) *J. nat. Cancer Inst.*, **15**, 493.
AMOS, D. B. AND WAKEFIELD, J. D.—(1958) *Ibid.*, **21**, 657.
KLEIN, G.—(1951) *Exp. Cell. Res.*, **2**, 518.
Idem and KLEIN, E.—(1956) *Nature*, **178**, 1389.
Idem AND RÉVÉSZ, L.—(1953) *J. nat. Cancer Inst.*, **14**, 229.
LAW, L. W., DUNN, T. B., BOYLE, P. J. AND MILLER, J. H.—(1949) *Ibid.*, **10**, 179.
MAZUREK, C. AND DUPLAN, J. F.—(1959) *Bull. Ass. franç. Cancer*, **46**, 119.
PUCK, T. T., MARCUS, P. I. AND CIECIURA, S. J.—(1956) *J. exp. Med.*, **103**, 273.
RÉVÉSZ, L.—(1955) *J. nat. Cancer Inst.*, **15**, 1691.—(1958) *Ibid.*, **20**, 1157.
RINGERTZ, N., KLEIN, E. AND RÉVÉSZ, L.—(1959) *Cancer*, **12**, 697.
SCHNEYER, C. A.—(1955) *Cancer Res.*, **15**, 268.
SCHREK, R.—(1936) *Amer. J. Cancer*, **28**, 389.
SCOTT, O. C. A.—(1957) *Brit. J. Cancer*, **11**, 130.
SHELTON, E. AND RICE, M. E.—(1958*a*) *J. nat. Cancer Inst.*, **21**, 137.—(1958*b*) *Ibid.*, **21**, 163.
SNELL, G. D.—(1955) *Transplant. Bull.*, **2**, 6.
VASILIEV, JU. and OLSHEVSKAJA, L. V.—(1958) *Voprosy onkologii*, **4**, 548.

EFFECT OF CHEMOTHERAPEUTICS ON THE NUCLEIC ACID METABOLISM OF TUMOURS
INCORPORATION OF ^{32}P INTO NUCLEIC ACIDS FOLLOWING TREATMENT WITH DEGRANOL

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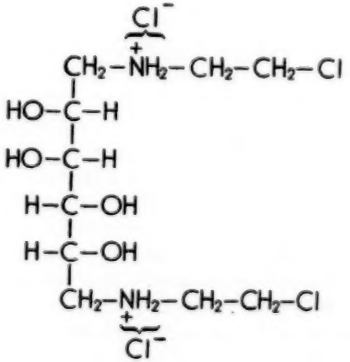
OUTSTANDING among the significant results achieved in recent years in the study of nucleic acids has been the recognition of the parallelism of their synthesis to mitotic cellular activity (Hershey, 1954 ; Healy *et al.*, 1956). Owing to their supreme significance in the metabolism of tumours, and in tumour therapy efforts are now concentrating on the development and utilization of agents which, directly or indirectly, act upon them (Skipper and Bennett, 1958 ; Shive and Skinner, 1958).

Chemotherapeutics, particularly those which act on the nucleic acids, are nitrogen mustard and its derivatives. One of them is the compound, first prepared by Vargha (1955) 1, 6-bis (β-chloroethylamino)-1, 6-dideoxy-D-mannitol-dihydrochloride, known as Degranol or BCM, the structural formula of which may be represented as follows :



For its tumour-inhibiting and haematological effects Degranol has been studied extensively by Kellner and co-workers (Kellner and Németh, 1956 ; Kellner, 1956 ; Németh, Kellner and Lapis, 1958 ; Lapis and Németh, 1956*a*, *b*) who found that

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the morphological changes to which the drug gives rise follow one another at fairly regular intervals: mitoses begin to be damaged 6 to 12 hours after the administration of a single dose; the peak effect involving extensive destruction takes from 48 to 72 hours to appear; and the restitutive stage sets in at about the 96th hour.

On the analogy of the earlier morphological investigations, we studied the dynamics of the effect Degranol exerts on the nucleic acid metabolism of tumours. The present paper reports the results of experiments undertaken to shed light upon the changes which a single dose of the drug brings forth in the incorporation of ^{32}P into the nucleic acid fractions and acid-soluble nucleotides of transplanted tumours.

MATERIALS AND METHODS

Experimental animals and their treatment with Degranol.—Inbred albino rats weighing 120 to 150 g. were inoculated subcutaneously with Guérin carcinoma and 14 to 16 days later given intravenously a single 50 mg./kg. dose of Degranol. Following this treatment they were decapitated at various times (24, 48, 72, 96 hours), but always after a 24-hour fast. The tumours they developed were removed and found to weigh from 8 to 12 g. Thin slices were cut off these tumours, fixed in formalin and embedded in paraffin for histology. Each tumour was worked up separately, mostly using its florid parts only. Whole tumours, however, were used in recovering ribonucleic acid (RNA) according to Pain and Butler (1957).

The ^{32}P was applied in the form of K_2HPO_4, after hydrolysis (to eliminate possible pyrophosphate) and neutralization. Each animal received a 100 μc/100 g. intraperitoneal dose of ^{32}P, 24 hours before decapitation.

Specific activity determination of acid-soluble nucleotides.—Hecht and Potter's (1956) method was employed in extracting the acid-soluble compounds. Tumour tissue (1 g.) was broken up thoroughly in a Potter-Elvehjem type homogenizer with 2 ml. of cold 0·6 N perchloric acid. The precipitate was centrifuged and washed with 0·2 N $HClO_4$. The supernatants were pooled and neutralized with KOH in the presence of phenol red as indicator. The $KClO_4$ was centrifuged off and the tissue inorganic orthophosphate (P_i) was removed by treatment with magnesia mixture (pH = 9) to which unlabelled phosphate had been added. The acid-soluble nucleotides were then adsorbed from an acid medium on active carbon and the adsorbate was centrifuged off, washed, and several times eluted with 50 per cent ethanol containing 2 per cent NH_3. The eluate was lyophilized and the residue taken up in water. Portions of the solution were used for radioactivity determination and for reading the nucleotide content in a Beckmann model DU spectrophotometer at 260 mμ in a 1 cm. quartz cuvette. Specific activity was expressed as cpm/1 $E_{260\ m\mu}$ (Pileri and Ledoux, 1957).

Measuring incorporation of ^{32}P into deoxyribonucleic acid (DNA), nuclear RNA (nRNA) and cytoplasmic RNA (cRNA).—Using a Potter-Elvehjem type homogenizer, the fragmented tumour tissue was homogenized in ten times its volume of cold 0·25 M sucrose containing 0·005 M of $CaCl_2$, and the homogenate was centrifuged in the cold at 600 *g* for 10 minutes, according to the method of Hogeboom, Schneider and Striebich (1952). Putting aside the cytoplasma supernatant, the precipitate was twice washed with 0·25 M sucrose, allowed to stand in 2 per cent

citric acid twice for half an hour each time, and washed twice with 2 per cent acetic acid (Nygaard and Rusch, 1955). All washings and centrifugations were done in the cold ; the latter invariably at 600 *g* for 10 minutes.

The nuclei isolated in the manner described were precipitated with 0·5 N $HClO_4$, and the cytoplasmic fraction was brought to 0·55 N $HClO_4$ concentration (Hurlbert and Potter, 1952). The precipitate was washed in the cold seven times with 0·2 N $HClO_4$ and twice with ethanol, whereafter it was extracted three times with a 3 : 1 mixture of ethanol and ether for 15 minutes at 60° C. The residue was washed with ether and dried *in vacuo*. From the dry powder the nucleic acids were isolated by the method of Davidson and Smellie (1952) : extraction was performed with 10 per cent NaCl (pH = 7) for 60 minutes at 100° C., and the procedure was repeated. The cRNA and nRNA were hydrolyzed with 0·3 N KOH for 16 hours at 30° C. In the nuclear fraction the DNA was separated from the RNA nucleotides by acidification following hydrolysis. The DNA was immediately centrifuged off, dissolved in dilute NH_4OH, purified by repeated precipitation, and finally dissolved in dilute NH_4OH.

Possible contaminating P_i was removed from the RNA nucleotides by means of magnesia mixture with unlabelled P_i added to it.

Portions of RNA and DNA fractions were used for radioactivity determinations and for reading the nucleic acid contents. The RNA content was determined by Mejbaum's (1939) orcinol reaction as modified by Ceriotti (1955), and the colour was read at 660 mμ on the Beckmann model DU spectrophotometer. The DNA content was determined by Dische's (1930) diphenylamine reaction as modified by Seibert (1940) and the colour was read at 600 mμ on the Beckmann model DU spectrophotometer. Specific activity was expressed as cpm/100 μg. of RNA-P and DNA-P, respectively.

Relative specific activity.—Following Smellie *et al.* (1955), the specific activity of the nucleotides and nucleic acids was referred to that of the P_i in the blood of the experimental animal and stated as relative specific activity, *i.e.* :

$$\text{Relative specific activity} = \frac{\text{specific activity of fraction studied}}{\text{specific activity of blood } P_i} \times 10000$$

Determination of specific activity of P_i in the blood.—The decapitated animal's blood was collected, and half its volume of trichloroacetic acid (20 per cent) was added to it. The mixture was centrifuged, and from the supernatant the P_i was isolated in the cold with magnesium mixture (pH = 9) as $MgNH_4PO_4$. This was recrystallized, and dissolved in dilute acid. Portions of the solution were then used to determine P_i by the method of Fiske and Subbarow (1925), as modified by Lohmann and Jendrassik (1926) and to measure radioactivity. Specific activity was expressed as cpm/100 μg. of P_i ; as mentioned above, to this was referred the specific activity of the nucleotides and nucleic acids, respectively, to be stated as the relative specific activity.

Isolation of RNA for heterogeneity tests was performed by the method of Pain and Butler (1957). The specific activity of the RNA so obtained was expressed as cpm/100 μg. of ribose.

Radiation count.—Portion of the solution to be studied was measured into an aluminium dish and there allowed to dry. Radioactivity was then measured under an end-window GM tube, with the aid of an Orion 1871 type counter.

RESULTS

Earlier animal experiments (Antoni *et al.*, 1958 ; Antoni, Vargha and Hidvégi, 1959) showed that for the isolation of RNA from liver labelled with ^{32}P *in vivo*, a 24-hour circulation time was the ideal. Even with subcellular particles this circulation time proved to be the best for labelling nucleic acids. Support for these findings has been provided by the work of Tyner, Heidelberger and LePage, (1953) on tumours, for which, in turn, we have established confirmation in respect of Guérin tumour.

TABLE I.—*Effect of Degranol on Relative Specific Activity of Tumour Nucleic Acids*

100 μc of ^{32}P/100 g. of body weight injected 24 hours before decapitation. The figures in brackets indicate the number of animals involved

Duration of treatment	Relative specific activity		
	DNA	nRNA	cRNA
Control	*3,950* (9) 3,530–4,200	*10,650* (10) 9,150–11,020	*8,300* (10) 6,950–8,750
24 hours	*1,850* (3) 1,410–2,460	*7,530* (3) 7,100–9,820	*8,050* (3) 7,050–8,250
48 hours	*1,520* (4) 1,120–1,860	*6,810* (4) 6,170–7,830	*7,250* (4) 6,810–7 750
72 hours Experiment *a*	*920* (7) 720–1,040	*4,950* (7) 4,610–6,050	*4,220* (7) 3,790–4,540
72 hours Experiment *b*	*2,900* (3) 1,940–3,720	*10,920* (3) 9,100–11,430	*9,210* (3) 7,110–9.850
96 hours	*4,420* (3) 3,840–4,960	*11,250* (3) 10,620–12,700	*8,820* (3) 8 070–9,440

Table I illustrates the effect Degranol exerted in our experiments on the incorporation of ^{32}P into tumoural nucleic acids. It shows that the drug caused their relative specific activity to decline at rates which followed a certain chronological regularity. The decrease was most marked in DNA : more than 50 per cent in 24 hours and reaching its minimum in 72 hours. It was less marked in nRNA and cRNA. At 96 hours after Degranol, *i.e.* at the time corresponding to the beginning of the restitutive stage as established by morphology, the relative specific activity of all the three nucleic acids studied was found to have returned to the level seen in the controls. In some cases, as early as 72 hours after treatment the relative specific activity was of the same order of magnitude in the experimental as in the control animals (cf. 72-hour experiment *b* in Table I). Histological examination showed the tumours of these animals to be in the restitutive stage.

Since in the current view the acid-soluble nucleotides are possible precursors of the nucleic acids our investigations were extended to the changes in their relative specific activity which follow treatment with Degranol. Table II illustrates the effect the drug had on the relative specific activity of acid-soluble nucleotides isolated simultaneously with nucleic acids and purified on active carbon. The data reveal that similar dynamic forces were at work in shaping

the relative specific activity of both sources: the purified acid soluble nucleotides and the nucleic acids. In some of our experiments the number of counts, registered 72 hours after Degranol, was found closely to approach the limit of errors; when averaging experimental results such counts were taken into consideration as non-radioactive values only.

TABLE II.—*Effect of Degranol on Relative Specific Activity of Acid-soluble Tumour Nucleotides*

100 μC of ^{32}P/100 g. of body weight injected 24 hours before decapitation. The figures in brackets inicate the number of animals involved

Duration of treatment	Relative specific activity
Control	*310* (10) 262–340
24 hours	*150* (3) 120–194
48 hours	*82* (4) 47–113
72 hours Experiment *a*	*20* (7) 0–73
72 hours Experiment *b*	*194* (3) 153–260
96 hours	*320* (3) 290–365

It was possible for us to verify our results in RNA fractions isolated by a different route (Pain and Butler, 1957).

The RNA was isolated from whole tumours, its specific activity was determined, and its heterogeneity studied (Antoni, Hidvégi and Lapis, 1960).

In Table III our experimental results are presented so as to demonstrate the *decrease* in the rate of ^{32}P incorporation following treatment with Degranol; in other words, the specific activity of the RNA in the tumour of the treated

TABLE III.—*Incorporation of ^{32}P into RNA Isolated from Whole Tumour According to Pain and Butler* (1957)

100 μC of ^{32}P/100 g. of body weight injected 24 hours before decapitation

Duration of treatment	Specific activity = $\frac{\text{control cpm/100 } \mu\text{g. of ribose}}{\text{treated cpm/100 } \mu\text{g. of ribose}}$
24 hours	*1·86* (3) 1·30–2·15
72 hours	*2·77* (6) 2·27–4·16
96 hours	*1·81* (3) 1·50–2·16

animal is referred to that in the tumour of the corresponding control animal. These data show that alterations induced by the drug in the rate of incorporation

of ^{32}P follow the same trend in RNA isolated by a different method, namely, from whole tumour.

DISCUSSION

Bodenstein (1947) was the first to study the effect of nitrogen mustard on nucleic acid metabolism. He found that the compound inhibited DNA synthesis, but assumed it had no inhibitory effect on the synthesis of RNA. (It would appear that the drug has no such effect on nuclei during mitosis, but does exert it in the interphase following mitosis.) Several authors hold similar views (Lowrance and Carter, 1950 ; Skipper *et al.*, 1951 ; Goldthwait, 1952). Harold and Zipporin recently found that mustard derivatives induced a transient inhibition of DNA synthesis in *Escherichia coli*, but allowed RNA and protein synthesis to continue unchanged.

Not unlike that of the other mustard derivatives, the initial effect of Degranol (between 6 and 24 hours) is to damage mitoses and bring down the total number of cell divisions. The peak effect falls between 48 and 72 hours, manifesting itself in extensive cellular and nuclear disintegration in the florid parts of the tumour, considerable expansion of necrotic areas, and the appearance of anomalous mitoses (cacomitoses) and multinuclear tumour changes are followed by the restitutive phase characterized by a cell population consisting predominantly of giant cells (Kellner and Németh, 1956 ; Kellner, 1956 ; Németh, Kellner and Lapis, 1958 ; Lapis and Németh, 1956*a*, 1956*b*).

On the evidence of our experimental results treatment with Degranol is followed by a decrease in the incorporation of ^{32}P into the nucleic acids and acid-soluble nucleotides of Guérin tumour. At the time it exerts its maximum effect the drug reduces the level of incorporation into DNA to about a quarter of the original. Incorporation into RNA and cRNA likewise shows decreases ; these however are less marked.

Our results, further, permit the statement that the changes in nucleic acid metabolism seen after Degranol are a dynamic process, the same as the morphological changes. In 24 hours a single dose decreases the relative specific activity of DNA by 50 per cent and reduces the total number of cell divisions. The drug exerts its maximum effect between the 48th and 72nd hour after input. This is the time when the morphological picture shows extensive cellular and nuclear disintegration and a progressive decline to the minimum of the relative specific activity of the nucleic acid fractions. This gradual decrease is particularly marked in DNA, a phenomenon which fairly corresponds to the extensive nuclear disintegration revealed by morphology. Conformity with the morphological picture is still more pronounced at 96 hours, when the relative specific activity of DNA, nRNA, and cRNA is observed to have returned to the level seen in the controls, coinciding with the appearance of multinuclear giant cells and amitoses in numbers so great as to be practically the only building stones of the entire florid tumours.

Conflicting interpretations of these cell forms are offered in the literature. Many authors have brought them into connection with regressive processes. The view, however, is gaining ever more adherents that they are very frequent and interesting forms of cell division. Kellner's studies (1952, 1960) also favour the idea that these kinds of cells have a decisive part to play in the restitutive processes that follow damages caused by chemotherapeutics. By our findings, at the time these cells come into prominence the level of relative specific activity of DNA rises

to that observed in the controls, which phenomenon seems to confirm that their appearance may actually be connected with processes progressive, and not regressive.

While in some cases, at 72 hours after Degranol the relative specific activity was the same in the experimental animals as in the controls, in others it showed the low level which characterized the maximal effect of the drug. Such differences, however, were invariably found to conform with the changes established by morphology. For, whenever the relative specific activity was as high as in the controls, the histological picture revealed changes typical of the restitutive stage ; conversely, whenever it was low, histology still showed marked nuclear destruction.

It seems safe to claim that our results offer the possibility of quantitatively characterizing the alterations and their individual phases, to which the mustard derivatives give rise in the tumour—and which hitherto could only be differentiated qualitatively on the strength of morphological changes—by measuring the incorporation of ^{32}P into the nucleic acids.

SUMMARY

The compound 1, 6-bis-(β-chloroethylamino)-1, 6-dideoxy-D-mannitol-dihydrochloride (Degranol ; BCM) has been studied for its effects on the nucleic acid metabolism of Guérin carcinoma transplanted into rats. A single dose of the drug was found to decrease incorporation of ^{32}P into the various nucleic acid fractions. At 72 hours after injection the decrease was most marked in DNA. It was less marked in nRNA and cRNA. At 96 hours, ^{32}P incorporation into all the three nucleic acids studied was seen to have returned to the levels observed in the control animals. Variations of a similar trend were noted in the acid-soluble nucleotide fractions isolated simultaneously with the nucleic acids.

The conclusion is drawn that the changes occurring in the nucleic metabolism of tumours represent a dynamic process, as do the morphological changes.

REFERENCES

Antoni, F., Hidvégi, E. J. and Lapis, K.—(1960) *Nature, Lond.*, **186**, 81.
Idem, Josepovits, G., Hidvégi, E. J. and Székessy-Hermann, V.—(1958) *4th Int. Congr. Biochem.*, Vienna.
Idem, Vargha, L. and Hidvégi, E. J.—(1959) *Acta physiol. Acad. Sci. hung.*, **16**, 1.
Bodenstein, D.—(1947) *J. exp. Zool.*, **104**, 311.
Ceriotti, G.—(1955) *J. biol. Chem.*, **214**, 59.
Davidson, J. N. and Smellie, R. M. S.—(1952) *Biochem. J.*, **52**, 594.
Dische, Z.—(1930) *Microchemie*, **8**, 4.
Fiske, C. H. and Subbarow, Y.—(1925) *J. biol. Chem.*, **66**, 375.
Goldthwait, D. A.—(1952) *Proc. Soc. exp. Biol. N.Y.*, **80**, 503.
Healy, G. M., Siminovitch, L., Parker, R. C. and Graham, A. F.—(1956) *Biochim. Biophys. Acta*, **20**, 425.
Hecht, L. I. and Potter, V. R.—(1956) *Cancer Res.*, **16**, 988.
Hershey, A. D.—(1954) *J. gen. Physiol.*, **38**, 145.
Hogeboom, G. H., Schneider, W. C. and Striebich, M. J.—(1952) *J. biol. Chem.*, **196**, 111.
Hurlbert, R. B. and Potter, V. R.—(1952) *Ibid.*, **195**, 257.

KELLNER, B.—(1952) *MTA Biol. és Orv. Tud. Osztályának Közleményei* (*Transactions of the Biological and Medical Section of the Hungarian Academy of Sciences*—Hungarian only), **7**, 435.—(1960) *Acta Un. int. Cancr.*, **16**, 80.—(1956) *Acta morph. hung.*, **7**, 215.

Idem AND NÉMETH, L.—(1956) *Z. Krebsforsch.*, **61**, 165.

LAPIS, K. AND NÉMETH, L.—(1956*a*) *Klin. Wschr.*, **34**, 864.—(1956*b*) *Brit. J. Cancer*, **10**, 719.

LOHMAN, K. AND JENDRASSIK, L.—(1926) *Biochem. Z.*, **178**, 419.

LOWRANCE, P. B. AND CARTER, C. E.—(1950) *J. cell comp. Physiol.*, **35**, 387.

MEJBAUM, W.—(1939) *Z. physiol. Chem.*, **258**, 117.

NÉMETH, L., KELLNER, B. AND LAPIS, K.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 879.

NYGAARD, O. AND RUSCH, H. P.—(1955) *Cancer Res.*, **15**, 240.

PAIN, R. H. AND BUTLER, J. A. V.—(1957) *Biochem. J.*, **66**, 299.

PILERI, A. AND LEDOUX, L.—(1957) *Biochim. Biophys. Acta*, **26**, 309.

SEIBERT, F. B.—(1940) *J. biol. Chem.*, **133**, 593.

SHIVE, W. AND SKINNER, C. G.—(1958) *Annu. Rev. Biochem.*, **27**, 643.

SKIPPER, H. E. AND BENNETT, L. L. Jr.—(1958) *Ibid.*, **27**, 137.

Idem, MITCHELL, J. H. Jr., BENNETT, L. L. Jr., NEWTON, M. A., SIMPSON, L. and EIDSON, M.—(1951) *Cancer Res.*, **11**, 145.

SMELLIE, R. M. S., HUMPHREY, G. F., KAY, E. R. M. AND DAVIDSON, J. N.—(1955) *Biochem. J.*, **60**, 177.

TYNER, E. P., HEIDELBERGER, C. AND LEPAGE, G. A.—(1953) *Cancer Res.*, **13**, 186.

VARGHA, L.—(1955) *Naturwissenschaften*, **42**, 582.

INDUCTION OF TUMOURS BY TANNIN EXTRACTS

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From the Chester Beatty Research Institute, Institute of Cancer Research Royal Cancer Hospital, London S.W.3

Received for publication February 1, 1960

TANNIC acid was shown to be carcinogenic to rats by Korpássy and Mosonyi (1950) and to have a synergistic effect with acetylamino-fluorene on the production of liver tumours in rats (Mosonyi and Korpássy, 1953 ; Korpássy, 1959).

Tannic acid is only one of a group of naturally occurring compounds which are known as vegetable tannins and are in general polyphenolic substances which have the ability to form a precipitate with gelatin under prescribed conditions. All these tannin extracts are mixtures of a number of components and no two are identical (White, Kirby, Knowles, 1952) although some compounds (e.g. gallic acid, ellagic acid, catechin) may be common to a number of extracts.

Some of these tannin extracts have been tested against both rats and mice with a view to determining the range of carcinogenic activity of this group of substances.

MATERIALS AND METHODS

Animals.—Rats of the August strain and stock and C_{57} black mice were used for the experiments.

Tannins.—The following extracts were used : sulphited Quebracho (*Schinopsis lorentzii*), Mimosa (*Acacia mollissima*), Myrtan (*Eucalyptus redunca*), Chestnut (*Castanea sativa* Mill.), Myrobalans (*Terminalia chebula* Retz), Valonea (*Quercus aegilops* L.) and Tannic Acid (B.D.H.). A single compound, fraction I from quebracho extract (Kirby, Knowles and White, 1953) has also been investigated. This substance was insoluble in water and was injected as a solution in 50 per cent propylene-glycol-water. In addition pure catechin and gallic acid have been employed. Tannin extracts usually contain 10–15 per cent moisture and 600 mg. of the extract was dissolved in water, adjusted to pH 6–6·5 with n/10 NaOH and the whole made up to 100 ml. with water. The solution was filtered if necessary before using and stored in a refrigerator. Rats were given a subcutaneous injection of 1 ml. each per week for 12 weeks and mice were given 0·25 ml. each per week for 12 weeks.

Feeding experiments.—Mice were allowed to drink 0·1 per cent solution of sulphited quebracho and of myrtan extracts for 3 months and then 0·5 per cent solutions of the same materials for 3 months. Mice drank these solutions in place of water and in fact, drank somewhat greater volume of the tannin solutions than other mice drank of water during the same period.

* British Empire Cancer Campaign Fellow.

RESULTS

Groups of 10 August rats were each injected with solutions of sulphited quebracho, mimosa, myrobalans and tannic acid. Sarcomas at the site of injection appeared about 1 year later in 2 August rats in each group of those injected with sulphited quebracho and mimosa extracts. Some of the animals in these groups had been killed earlier to look for damage to the liver, but none was observed. Both tumours produced by these extracts have been transplanted and have now reached the 90th generation. The original transplants required about 6 weeks to grow to a size of about 2 cm. diameter but after about 5 generations this period was reduced to 12 days. Tannic acid and myrobalan extracts had no observable effects on August rats.

Mice.—A wider group of tannin extracts has been used upon mice and the results are summarized in Table I.

TABLE I.—*Group of Tannin Extracts Used Upon Mice*

	Extract	Sarcomas	Liver tumours
Stock mice—			
	Myrtan	5	7
	Quebracho	8	5
	Mimosa	2	9
	Tannic acid	0	7
	Myrobalans	0	4
	Chestnut	0	4
	Valonea	0	1
C_{57} *mice*—			
	Quebracho (40)	2	0
	Chestnut (20)	0	0

Some lymphomas were also observed in these groups but they are of little significance, since they occur spontaneously in older stock mice.

Feeding experiments.—0·1 per cent solution of sulphited quebracho and of myrtan in water (pH not adjusted and was about 4·5) were fed to mice during 3 months and then 0·5 per cent solutions were fed for a further 3 months. No adverse effects were noticed in any of the animals during 1 year.

Histology.—Micro-photographs of sections of some of the tumours obtained by injecting tannin extracts into rats and mice are shown in Fig. 1–8. Fig. 1–4 show different types of sarcomata induced by quebracho, sulphited quebracho and mimosa extracts and Fig. 5–8 show liver tumours induced by myrtan and chestnut extracts and sarcomata by myrtan extract.

DISCUSSION

The effects produced in these experiments have been with minimum amounts of the tannin extracts. Korpássy and Mosonyi (1950) produced hepatomata with up to 9950 mg. of tannic acid/kg. body weight by injection, while in the experiments described a carcinogenic effect was observed after a total of 350 mg. of extract/kg. had been injected into the rat and 750 mg./kg. into the mouse.

The effect is not due to ulceration which was observed in some preliminary injections with tannic extracts and the dose was lowered so that no (or very little) ulceration was observed. Chestnut tannin extract was by far the most damaging

in this respect but no sarcomata resulted from injection of this material. While only a small number of tannin extracts have been investigated a correlation can be noted between the tumour resulting and the type of extract injected.

Tannins may be classified quite generally into 2 types: (1) hydrolysable tannins which on heating with mineral acids yield glucose, possibly other sugars and gallic and related polyphenolic acids; (2) condensed tannins which produce intractable red precipitates on heating with mineral acids. While little of the chemical nature of these latter type of tannins is known, polyphenolic residues are present and may be linked through polyhydroxyflavan nuclei.

Of the tannins used in this investigation quebracho, mimosa and myrtan extracts are of the condensed variety while myrobalans, chestnut and valonea extracts are hydrolysable tannins. While all the extracts induced some liver tumours, only the condensed tannins produced sarcomata at the site of injection. Moreover, when 2 condensed tannins were fed through the drinking water no lesions were observed, in agreement with the results of Korpássy and Mosonyi (1950) who observed no liver tumours by feeding tannic acid.

Although tannins are complex mixtures it was found possible to induce a sarcoma in the mouse by injection of a single component (judged by a paper chromatography) from quebracho extract and possibly only a small number of the components present in any one extract may be carcinogenic.

The production of a sarcoma at the site of injection by condensed tannins is in agreement with the experiments of Armstrong, Clarke and Cotchin (1957) who suggested that condensed tannins were held at the site of injection more firmly than hydrolysable tannins.

Gallic acid has been reported not to have any carcinogenic activity and catechin (3 : 5 : 7 : 3′ : 4′-penta-hydroxyflavan, the basis of some of the tea tannins) is negative in mice at the levels tried in these experiments.

It is difficult to speculate on the carcinogenic activity of tannin extracts particularly when relatively little is known of their chemical structure and when substances of such a wide difference of structure as 4-dimethylamino-azo-benzene (Kinosita, 1937), thioacetamide (Rather, 1951), dimethylnitrosamine (Magee and Barnes, 1956) and ethionine (Farber, 1956) all produce liver tumours in the rat or the mouse. However, the two important features of tannin reactivity which are known are the reaction with proteins which results in the formation of leather and the ability to complex with metals, of which the complex between tannic acid and iron salts is the most well known. Whether either or both of these reactions are of importance in the carcinogenic activity must remain for future experiments.

SUMMARY

Tumours have been induced in rats and mice by subcutaneous injections of various tannin extracts. Condensed tannins evoked sarcomata at the site of injection as well as liver tumours, but liver tumours only were produced by extracts of hydrolysable tannins.

This work was carried out during the tenure of a British Empire Cancer Campaign Research Fellowship.

I wish to thank Professor A. Haddow, F.R.S. for his help and advice, the late Professor E. Horning and Dr. L. Foulds for the pathology, Mr. R. T. Charles for

assistance with the animal experiments, Mr. S. R. Scarfe for the preparation of the histological sections, Mr. K. Moreman for the photographs and Miss G. E. Adams for technical assistance. The tannin extracts were kindly provided by Dr. T. White and Dr. H. J. C. King of the Forestal Laboratories, Harpenden.

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REFERENCES

ARMSTRONG, D. M. G., CLARKE, E. G. C. AND COTCHIN, E.—(1957) *J. Pharm., Lond.*, **9**, 98.
FARBER, E.—(1956) *Cancer Res.*, **16**, 142.
KINOSITA, R.—(1937) *Trans. Jap. path. Soc.*, **27**, 665.
KIRBY, K. S., KNOWLES, E. AND WHITE, T.—(1953) *J. Soc. Leath. Tr. Chem.*, **37**, 283.
KORPÁSSY, B.—(1959) *Cancer Res.*, **19**, 501.
Idem AND MOSONYI, M.—(1950) *Brit. J. Cancer*, **4**, 411.
MAGEE, P. N. AND BARNES, J. M.—(1956) *Ibid.*, **10**, 114.
MOSONYI, M. AND KORPÁSSY, B.—(1953) *Nature, Lond.*, **171**, 791.
RATHER, L. J.—(1951) *Johns Hopk. Hosp. Bull.*, **88**, 38.
WHITE, T., KIRBY, K. S. AND KNOWLES, E.—(1952), *J. Soc. Leath. Tr. Chem.*, **36**, 148.

EXPLANATION OF PLATES

All sections were stained with haematoxylin and eosin.

FIG. 1.—Fibrosarcoma in the mouse induced by injecting a single component from quebracho extract. This tumour shows a tendency to differentiate in the direction of the fibrous connective tissue. (×130.)

FIG. 2.—Spindle cell sarcoma in the mouse, induced by injecting sulphited quebracho extract. (×130.)

Fig. 3.—Spindle cell sarcoma in the rat induced by injecting mimosa extract. This material was from a transplant of the 15th generation. (×150.)

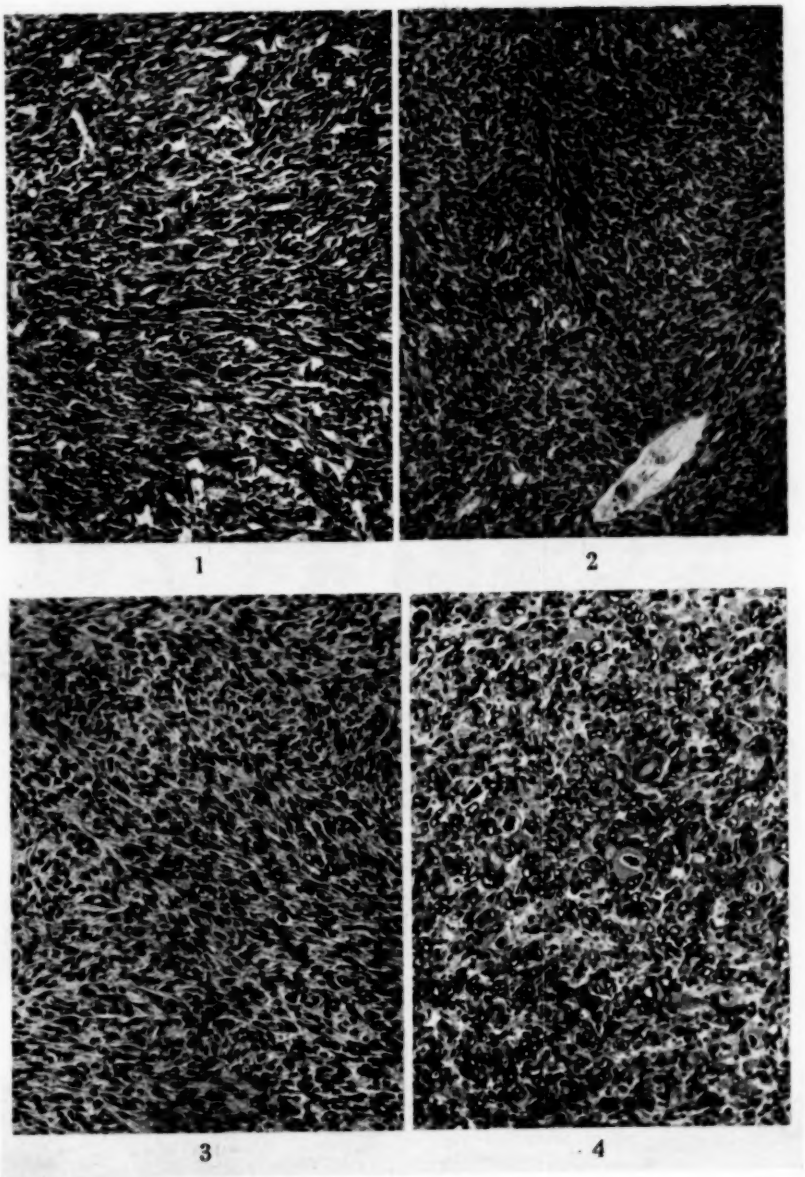
FIG. 4.—Pleomorphic sarcoma in the rat induced by injecting sulphited quebracho extract. Many giant cells are present. (×130.)

FIG. 5.—Early hepatoma in the mouse induced by injecting myrtan extract. (×130.)

FIG. 6.—Hepatoma in the mouse induced by injecting chestnut tannin extract. (×150.)

FIG. 7.—Mixed cell sarcoma in the mouse induced by injecting myrtan extract. (×150.)

FIG. 8.—Spindle cell sarcoma in the mouse induced by injecting myrtan extract. (×150.)



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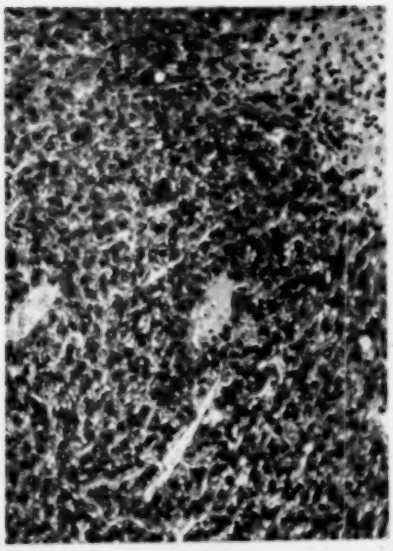

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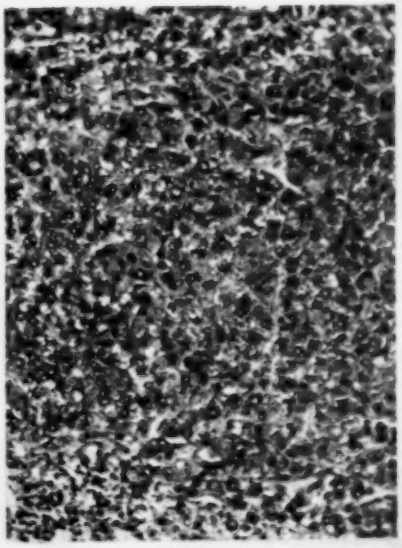

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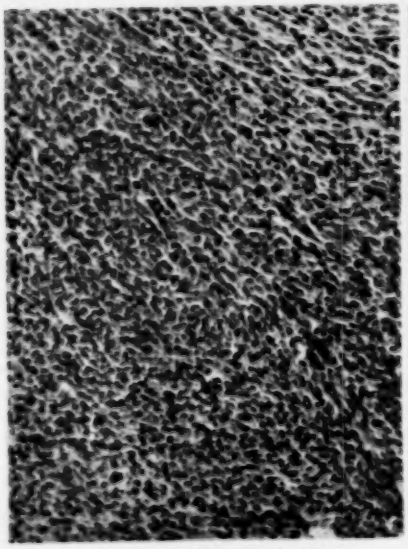

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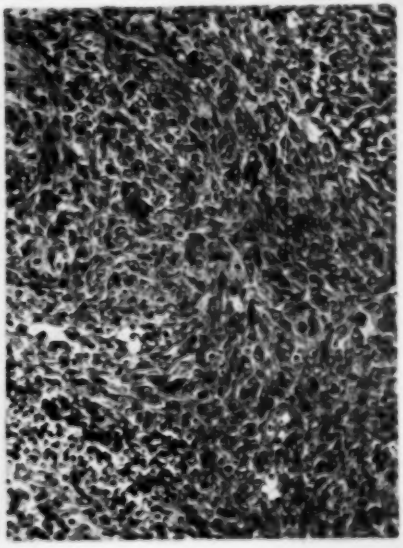

8

SURVEY OF PAPERS.

MUIR describes the morbid anatomical features of a series of lung, tracheal and laryngeal cancers occurring in Singapore, comparing the tumours and their incidence with those seen elsewhere (p. 1).

STOCKS and DAVIES have studied chemically and spectrographically garden soils in North Wales, Cheshire and two localities in Devon, and find correlations between the amounts of certain constituents and the frequency of cancer of the stomach (p. 8).

HANBURY reports a study of 50 cases of discrete secondary tumours of the heart, the factors considered being the distribution of tumours within the heart, the types of associated primary tumours, and the incidence of associated metastases in other organs (p. 23).

ENGLANDER and SARANGI find that Thio-TEPA has a marked, but temporary, growth inhibiting effect in some cases of carcinoma of the ovary (p. 28).

DALE describes a method for isolating the epithelial cells from human colonic mucosa and from carcinoma of the colon (p. 45).

GRIESBACH and PURVES find a high incidence of basophil adenomata in the hypophyses of rats of both sexes examined 15 months or more after gonadectomy, no factor other than a long-continued deficiency of gonadal hormones in an otherwise healthy rat being necessary for inducing them (p. 49).

PRESNOV describes the histological and histochemical changes accompanying regression induced in three transplanted rat sarcomas by the alkylating agents sarcolysine, dopan and ethylene-iminobenzoquinone (p. 60).

PORTER describes and compares the histological changes seen in rabbits suffering from secondary irradiation disease and runt disease (p. 66).

CARR finds that the MH2 virus of reticuloendothelioma will also induce kidney carcinoma in very young chicks. The tumours are only found if the host survives for several weeks and seem analagous to those induced by the ES4 virus (p. 77).

MILLER reports the results obtained in four different strains of mice inoculated as newborn or as adults with filtered extracts prepared from thymus, spleen and lymph nodes of mice with either spontaneous or induced leukaemias (p. 83).

MILLER has investigated the effect of thymectomy and thymus grafting on leukaemogenic activity of cell-free leukaemic extracts. He concludes that the potentiality for leukaemia development is still present in inoculated thymectomized hosts for many months after thymectomy and that thymus grafting at any time will express this potentiality in full (p. 93).

POWELL describes a fluid culture medium which supports the growth of Ehrlich carcinoma ascites tumour cells *in vitro*. The medium is suitable for short term assays of the effects of cytotoxic agents on the cultivated cells, since they can be quantitatively recovered for enumeration (p. 99).

LEWIS and PLAICE have estimated the β-glucuronidase activity in patients with cancer of the bladder and in a large number of controls. They present data which appear to correlate the significantly increased 24-hour output of the enzyme in bladder cancer with the increased volume of urine excreted (p. 106).

O'GORMAN and MIKULSKA report experiments which have shown, by means of graduated absorption of iso-antisera, that suspensions of the ascites tumour Sa 1 are markedly deficient in the H-2 antigens E and K, but not deficient in antigen D. They suggest that these deficiencies are connected with the lack of sensitivity of Sa 1 to cytotoxic antibody and the ease with which the growth of the tumour may be enhanced (p. 121).

SEELIG and RÉVÉSZ have studied the influence of a large number of irreversibly damaged tumour cells upon the subsequent proliferation of a small, admixed viable cell fraction, in diffusion chambers inserted into the peritoneal cavity of mice. They find that viable cells admixed to heavily irradiated material of the same or different tumours or to liver tissue grow more rapidly than the same number of viable cells cultured alone (p. 126).

HIDVÉGI, ANTONI and LAPIS report an experimental investigation into the changes in the incorporation of ^{32}P into the nucleic acid fractions and acid-soluble nucleotides of the Guérin carcinoma in rats which result from a single dose of Degranol (p. 139).

KIRBY has induced tumours in rats and mice by subcutaneous injections of various tannin extracts. He finds that condensed tannins evoke sarcomata at the site of injection as well as liver tumours, but that liver tumours only are produced by extracts of hydrolysable tannins (p. 147).

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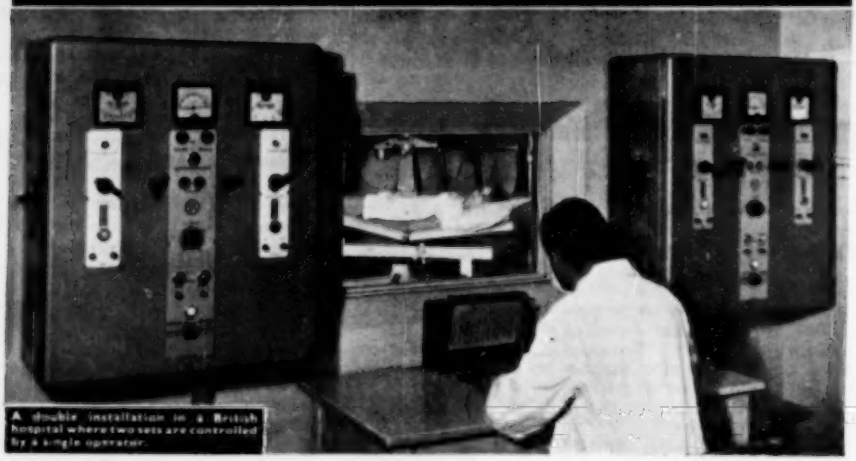

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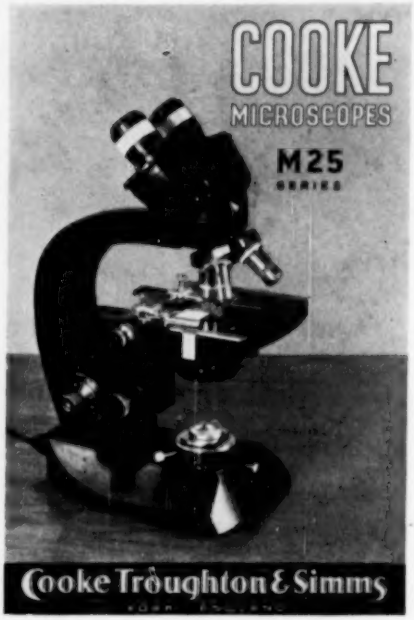

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Due to illness, Professor Neubauer was unable to complete his task in its final stages, and this was undertaken by Miss Phyllis M. Coombs, of the library staff of the Chester Beatty Research Institute. The work throughout was supported by the British Empire Cancer Campaign, which has also made a substantial contribution towards the cost of publication. The *Bibliography* will be of lasting value not only to those engaged in cancer research, but also to a much wider circle of interests in chemistry, industry, and experimental pathology.

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By Otto Neubauer

This is a survey of the literature of cancer producing compounds up to and including 1947 compiled by Professor Otto Neubauer, late of Munich, who was well known in the fields of intermediary metabolism and chemical pathology.

Leaving Germany in 1939, he then settled in Oxford, where he remained until his death in 1957. During his stay in this country, his friendship with the late Sir Ernest Kennaway led the latter to encourage him to compile a much needed bibliography of cancer produced by pure chemical compounds, and the present volume is the result.

Due to illness, Professor Neubauer was unable to complete his task in its final stages, and this was undertaken by Miss Phyllis M. Coombs, of the library staff of the Chester Beatty Research Institute. The work throughout was supported by the British Empire Cancer Campaign, which has also made a substantial contribution towards the cost of publication. The *Bibliography* will be of lasting value not only to those engaged in cancer research, but also to a much wider circle of interests in chemistry, industry, and experimental pathology.

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